Lanthipeptide engineering: non-canonical amino acids, click chemistry and ring shuffling

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Contents

CHAPTER 1

General introduction

1. Overview of lanthipeptides

Ribosomally synthesized and post-translationally modified peptides (RiPPs) represent an important class of natural products which are found in all three domains of life. They are genetically encoded and initially synthesized as linear precursor peptides consisting of two obligatory regions (a leader and a core peptide).[1] A leader peptide is usually located at the N-terminus of precursor which is important for keeping peptide inactive to protect the host and for recognition by post-translational modification (PTM) enzymes and transporter. The C-terminal core peptides undergo numerous PTMs including dehydration (e.g. lanthipeptides, thiopeptides), cyclodehydration (e.g. bottromycins), epimerization (e.g. proteusins), head-to-tail cyclization (e.g. AS-48) or internal cyclization with a peptide chain passing through a ring structure (lassopeptides). The extensive post-translational modifications endows RiPPs with specific target recognition, decreased conformational flexibility and increased metabolic stability compared to linear peptides. There are more than 20 RiPP classes reported up to date, which are subclassified depending on their characteristic structural and biosynthetic features.[1] A prominent class of RiPPs is constituted by lanthipeptides, lanthionine-containing peptides, among which those with antimicrobial activity are called lantibiotics.[1] Lantibiotics are antimicrobial peptides harbouring unusual post-translationally modified amino acid residues such as dehydroalanine (Dha) and dehydrobutyrine (Dhb), lanthionines (Lans) and methyllanthionines (MeLans).[2] Some lantibiotics have been considered as lead structures for therapeutic use.[3-5] MU1140[6] and NAI-107[7] are in late pre-clinical trials against Gram-positive bacteria. Duramycin has completed phase II clinical trials for the treatment of cystic fibrosis.[8] NVB302, a derivative of the lantibiotic actagardine, has completed phase I clinical trials for the treatment of *Clostridium difficile*.[9,10]

1.1. Classification of lanthipeptides

Lanthipeptides are divided into four classes according to the PTM enzymes for ring formation (Figure 1).[11] In class I lanthipeptides, the dehydration of serine and threonine is carried out by dehydratase LanB. The dehydrated residues are then cyclized with cysteine catalyzed by cyclase LanC. The flexible elongated secondary structure of class I lanthipeptides (eg. nisin, gallidermin, subtilin, and Pep5) play an essential role in their antimicrobial activity by binding to lipid II and/or in the pore formation.[12,13] For class II lanthipeptides, there is a single bifunctional synthetase (LanM) that performs both dehydration and cyclization. LanM contains an N-terminal dehydration domain and a C-terminal LanC-like cyclase domain. The N-terminal dehydration domain does not display any sequence homology with other enzymes.[11] For class III and IV lanthipeptides, dehydration and cyclization are catalyzed by a single trifunctional synthetase (LanKC for class III and LanL for class IV).[11] LanKC and LanL show the same

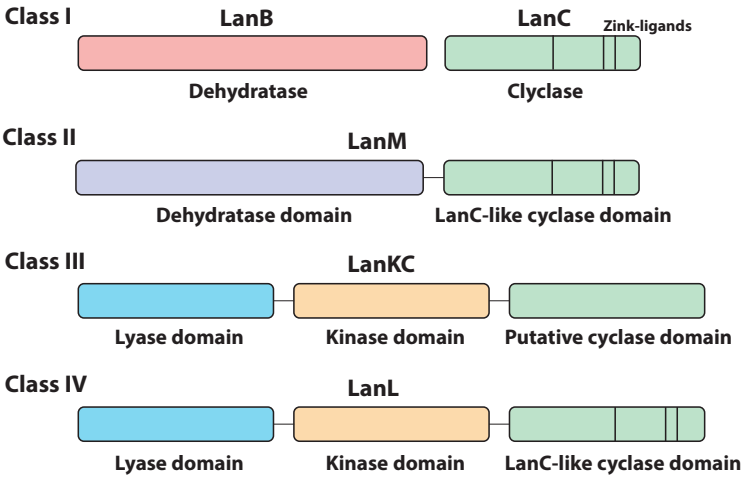

Figure 1. Schematic representation of the four lanthipeptide classes of lanthionine synthetases. (adapted from Knerr et al.[11])

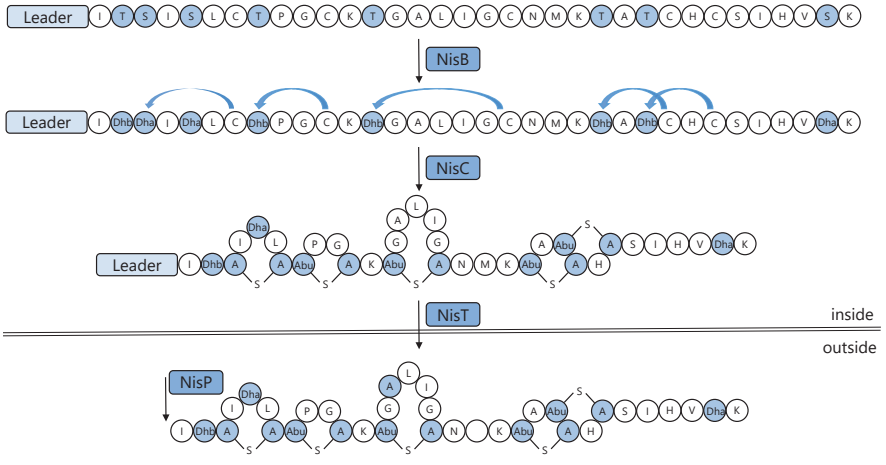

Figure 2. Maturation of nisin.

dehydration mechanism processed by an N-terminal lyase domain and a central kinase domain, but they differ in their C-terminal cyclase domains. The cyclase domain of LanKC lacks the characteristic zinc binding found in LanC and LanC-like cyclases.[14]

1.2. Nisin

Nisin is the first discovered and the best studied lantibiotic and is produced by *Lactococcus lactis*.[15] It has been used as a powerful and safe preservative against food spoilage bacteria for over 50 years.[16,17] Besides its preservative properties, nisin is effective

against many antibiotic resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE).[18] Nisin contains one lanthionine and four methylanthionine rings. The first two rings (AB) form the lipid II recognition site. By binding to the peptidoglycan precursor lipid II, the synthesis of cell wall is inhibited. The last three rings including the hinge region (C-E) constitute the membrane insertion domain. After binding to lipid II, nisin can form lipid II-nisin hybrid pores in thre target cell membrane.[19]

The biosynthesis of nisin is encoded by a cluster of 11 genes *nisABTCIPRKFEG*.[19,20] The genes *nisR* and *nisK* encode a two component regulatory system for regulating the immunity and production of nisin. In the presence of nisin, NisK phosphorylates itself and transfers the phosphate moiety to NisR which triggers the transcription of *nisABTCIP* and *nisFEG*. The *nisA* gene encodes a linear precursor nisin (57 aa), which is composed of a leader peptide (23 aa) and a core peptide (34 aa). After ribosomal synthesis of the precursor peptide, the unmodified prenisin is processed by the specific modification machinery (Figure 2). The precusor peptide will be dehydrated and cyclized by the modification enzymes to form the lanthionine rings. Subsequently, the modified prenisin is transported by the ABC-type transporter NisT, and then the leader peptide is cleaved off by the extracellular protease NisP to liberate active nisin. *nisI* and *nisFEG* encode immunity proteins that protect the host from nisin.

It has been reported that the PTM enzymes NisBC have a relaxed substrate specificity.[21-23] Nisin regulation genes are widely used to control gene expression. A lot of gene expression systems have been constructed for *L. lactis* and other Gram-negative bacteria.[24-29] The nisin-controlled gene expression system (NICE) is the most successful and widely used.[30-33] It was first constructed by Kuipers et al. based on the autoregulation mechanism of nisin biosynthesis.[34] A two plasmid expression system was subsequently developed for high level expression of proteins from different origins for various applications. One plasmid with nisin modification enzymes and the other plasmid carrying the gene of interest are both under control of nisin promoter P*nisA*. A wide range of clinical relevant peptides (e.g. nukacin ISK-1, enkephalin, somatostatin, and angiotensin) have be successfully modified and secreted by this system.[35-37] Another tightly-controlled expression system in *L. Lactis* is the zinc-regulated expression system (Zirex) which was constructed by introducing the streptococcal promoter P*czcD* together with the repressor SczA.[29] This system is able to achieve a high expression level of proteins that is comparable to that of the NICE system. Moreover, a cross expression system by combining zinc inducible promoter P*czcD* with nisin inducible promoter P*nisA* was developed, enabling the independent expression of different proteins at different times. Overall, the broad substrate tolerance of NisBTC and the regulated gene expression systems make it possible to efficiently engineer both lantibiotics and non-lantibiotic peptides with enhanced functionalities in *L. lactis*.[38]

2. Lantibiotic engineering

Engineering of lantibiotics is well feasible as they are gene-coded and can be readily manipulated with few genetic manipulations. Over the last few decades, various synthetic and biosynthetic strategies have been developed to produce lantibiotic derivatives with improved therapeutic properties (e.g. activity, stability, and solubility) and/or altered antimicrobial spectrum which make them suitable for applications.[38,39] In addition, lantibiotics engineering can also help us to understand the mode of action, structure-activity relationship, substrate tolerance, and biosynthesis machinery and to identify essential amino acids.[5] To meet the significant challenges of the structural complexity of lantibiotics, it is convenient that a wide range of tools exist. Here, lantibiotic derivatives generated from incorporating non-canonical amino acids (ncAAs) and chemical modification via click chemistry are introduced.

2.1 Incorporation of non-canonical amino acids into lantibiotics

ncAAs in lantibiotics can contribute to biological activity and structural stability. Incorporation of ncAAs is a promising strategy to broaden the structural diversity of ribosomal peptides.[40] The insertion of various ncAAs with unique features during translation represents a novel level of chemical diversification and it can expand the scope of ribosomal peptide synthesis based on the standard set of 20 canonical amino acids (cAAs).[40,41] The ribosomal incorporation of ncAAs can be achieved by two main approaches: selective pressure incorporation (SPI) and stop-codon suppression (SCS).

The first approach, termed SPI, is also called residue-specific incorporation.42 This method allows for the global replacement of certain cAAs by their related isostructural non-canonical analogues. ncAAs can be incorporated at high level by using an auxotrophic host strain that is unable to synthesize the targeted cAA. Odal and co-workers were the first to propose the use of SPI as tool to incorporate 11 analogues of methionine, proline and tryptophan into a two-component lantibiotic lichenicidin (Bliα and Bliβ) using the lichenicidin biosynthetic machinery in auxotrophic *Escherichia coli* strains (Table 1).[43] The methionine analogue Hpg-containing lichenicidin was coupled to fluorescein as an example of post-biosynthetic modifications of a lantibiotic.[43] This study indicated that the recombinant expression of bioactive peptides with various ncAAs could enable the design of novel lantibiotic. An evolutionarily adapted host strain *E. coli* MT21 was developed to incorporate a cellular toxic tryptophan analogue [3,2] Tpa (Table 1).[44] For the first time, it provided a proof-of-principle for the application of an evolutionarily adapted strain for the production of novel ncAAs-containing lantibiotics. The insertion of tryptophan analogues and proline analogues into nisin by SPI was achieved using a tryptophan-auxotrophic *L. lactis* strain[45] and a proline-auxotrophic *E. coli* strain[46], respectively (Table 1).

The second method, SCS, is also named site-specific incorporation.[47] It involves the use of an orthogonal aminoacyl-tRNA synthetase/suppressor tRNA (aaRS/tRNA) pair that is able to charge the targeted ncAA in response to an amber stop codon, which is then directly incorporated by the ribosomal translation machinery into peptides. This method is ideal for introducing specific point mutations into peptides. Genetic manipulation of the target sequence is required and incorporation of more than one ncAA is challenging. Van der Donk and co-workers first demonstrated the incorporation of ncAAs into lantibiotics using a stop codon suppression method. As a first proof of principle, *p*-benzoyl-L-Phe was incorporated into prochlorosin A3.2 by introducing an amber stop codon (tag) at the position of F26 and an orthogonal tRNA synthetase/tRNACUA pair (Table 1).[48] The incorporation of hydroxyl acids into the precursor peptides lacticin 481 and nukacin ISK-1 was achieved in *E. coli* by using an orthogonal pylRS/tRNAPyl pair, resulting in the connection of the leader peptide and core peptide via an ester bond that is readily cleaved by alkaline hydrolysis. It proved to be a successful method for *in vivo* production and subsequent leader peptide removal of lacticin 481 and nukacin ISK-1 analogues (Table 1).[49] Using the same platform, phenylalanine analogues were successfully incorporated at three positions of lacticin 481 and nisin (Table 1).[50] One of the lacticin 481 variants, i.e. Trp19-*o*-NO2Phe showed better antimicrobial activity than WT. The insertion of ncAAs into deoxycinnamycin was achieved for the first time in *Steptomyces albus* by using the orthogonal pylRS/tRNAPyl pair (Table 1).[51]

Table 1. Lantibiotic derivatives and their characteristics.

Lantibiotic	Mutation	Position	ncAAs	Method	Biological Activity	Ref.
Bliα	No	28	Aha	SPI	Same	43
Bliα	No	28	Hpg	SPI	Reduced	43
Bliα	No	28	Nle	SPI	Similar	43
Bliα	No	28	Eth	SPI	Similar	43
Bliα	No	13/29	(4*R*-OH)Pro	SPI	ND	43
Bliα	No	13/29	(4*R*-F)Pro	SPI	ND	43
Bliα	No	13/29	(4*S*-F)Pro	SPI	ND	43
Bliα	No	13/29	(S)Pro	SPI	ND	43
Bliβ	No	9	(4-F)Trp	SPI	ND	43
Bliβ	No	9	(5-OH)Trp	SPI	ND	43
Bliβ	No	9	(7-Aza)Trp	SPI	ND	43
Bliβ	No	10	[3,2]Tpa	SPI	Similar	44
Nisin	No	9	*trans*F	SPI	ND	46,48
Nisin	No	9	*cis*F	SPI	ND	46,48
Nisin	No	9	*trans*OH	SPI	ND	46,48
Nisin	No	9	*cis*OH	SPI	ND	46,48
Nisin	No	9	*trans*Me	SPI	ND	46,48
Nisin	No	9	*cis*Me	SPI	ND	46,48
Nisin	I1W	1	5FW	SPI	2 fold lower	45

Lantibiotic	Mutation	Position	ncAAs	Method	Biological Activity	Ref
Nisin	I1W	1	5HW	SPI	4 fold lower	45
Nisin	I1W	1	5MeW	SPI	ND	45
Nisin	I4W	4	5FW	SPI	2 fold lower	45
Nisin	I4W	4	5HW	SPI	ND	45
Nisin	I4W	4	5MeW	SPI	ND	45
Nisin	M17W	17	5FW	SPI	ND	45
Nisin	M17W	17	5HW	SPI	32 fold lower	45
Nisin	M17W	17	5MeW	SPI	ND	45
Nisin	V32W	32	5FW	SPI	ND	45
Nisin	V32W	32	5HW	SPI	ND	45
Nisin	V32W	32	5MeW	SPI	ND	45
Nisin	I4tag	4	BocK	SCS	Reduced	52
Nisin	K12tag	12	BocK	SCS	Reduced	52
Nisin	I4V/S5tag/L6G	5	*m*-BrPhe	SCS	Reduced	50
Nisin	S3tag	3	Fluoto-pAcF	SCS	ND	53
Nisin	S3tag	3	Chloro-pAaF	SCS	Reduced	53
Nisin	S5tag	5	pAcF	SCS	ND	53
Nisin	S5tag	5	pAzF	SCS	ND	53
Nisin	S5tag	5	procK	SCS	ND	53
Nisin	T8tag	8	Chloro-pAaF	SCS	Reduced	53
Nisin	T13tag	13	Chloro-pAaF	SCS	Reduced	53
Nisin	S3tag/C7-11-19-26-28A/S29A	3	Chloro-pAaF	SCS	ND	53
Nisin	S3tag/C11-19-26-28A	3	Chloro-pAaF	SCS	ND	53
Nisin	S3TAG/C7-11-19-26-28A	3	C13-labelled Chloro-pAaF	SCS	ND	53
Nisin	S3TAG/C11-19-26-28A/S29A	3	C13-labelled Chloro-pAaF	SCS	ND	53
Lacticin 481	N15R/W19tag	19	*m*-BrPhe	SCS	Same	50
Lacticin 481	N15R/W19tag	19	*o*-ClPhe	SCS	Same	50
Lacticin 481	N15R/W19tag	19	o-NO2Phe	SCS	Increased	50
Lacticin 481	N15R/F21tag	21	o-NO2Phe	SCS	Same	50
Lacticin 481	N15R/F23tag	23	*o*-ClPhe	SCS	Same	50
Lacticin 481	A-1I/K1tag	1	Boc-1	SCS	ND	49
Lacticin 481	A-1I/K1tag	1	Boc-HO-1	SCS	Reduced	49
Lacticin 481	A-1I/K1tag	1	H-Phe(3-Br)-OH	SCS	ND	49
Lacticin 481	A-1I/K1tag	1	HO-Phe(3-Br)-OH	SCS	Reduced	49
Lacticin 481	A-1I/K1tag	1	H-Tyr(propargyl)-OH	SCS	ND	49
Lacticin 481	A-1I/K1tag	1	HO-Tyr(propargyl)-OH	SCS	Reduced	49
Nukacin	A-1I/K1tag	1	Boc-HO-1	SCS	Reduced	49
Deoxycinnamycin	R2tag	2	Alk	SCS	2-fold better	51
Deoxycinnamycin	R2tag	2	Cyc	SCS	ND	51
Deoxycinnamycin	F10tag	10	Alk	SCS	2-fold lower	51
Deoxycinnamycin	F10tag	10	Cyc	SCS	ND	51
Deoxycinnamycin	F10tag	10	Boc	SCS	ND	51
Prochlorosin A3.2	F26tag	26	*p*-benzoyl-L-Phe	SCS	ND	48
Prochlorosin A3.2	F26tag	26	*p*-benzoyl-L-Phe	SCS	ND	48

No: no mutation; tag: amber stop codon; SPI: selective pressure incorporation; SCS: stop-codon suppression; ND: not determined.

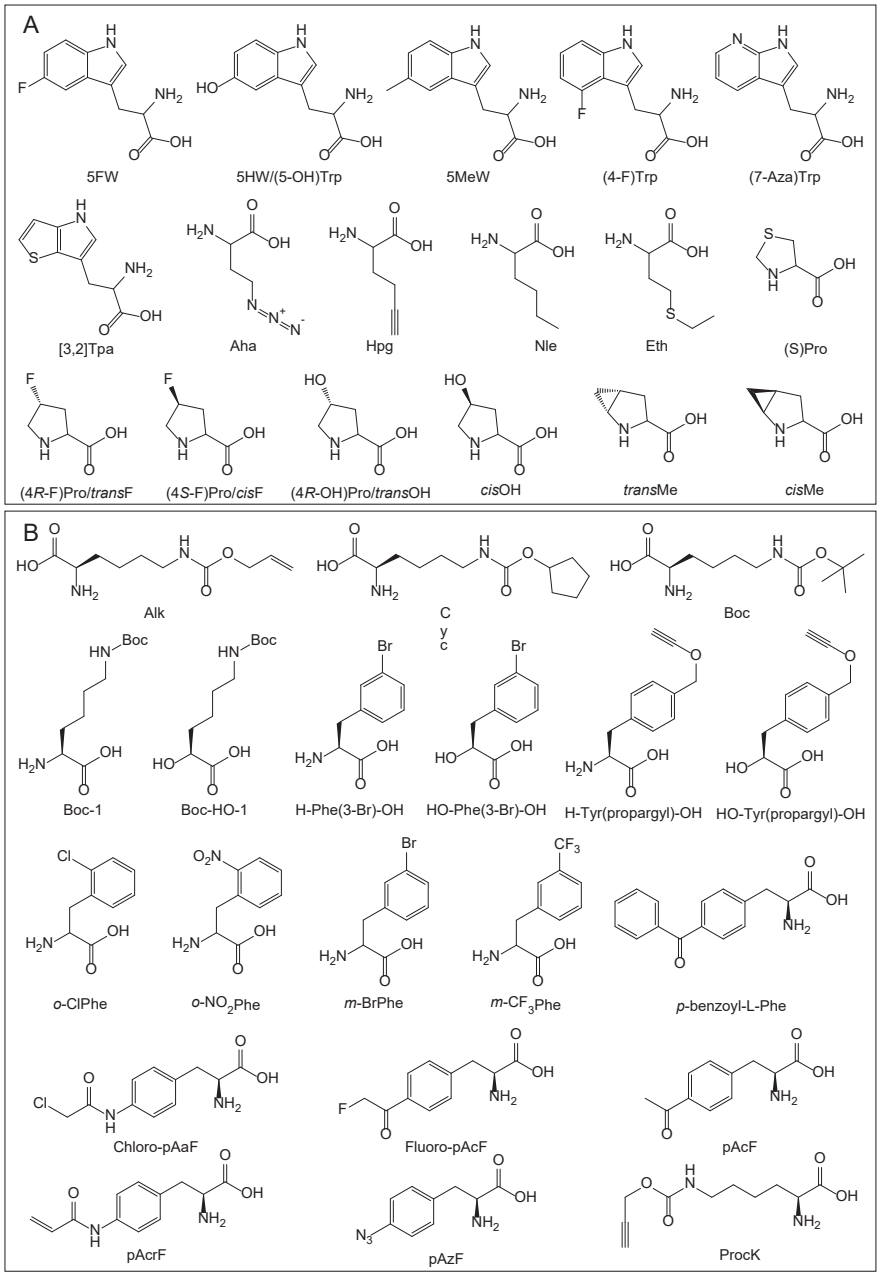

Figure 3. Non-canonical amino acids incorporated into Lantibiotics *in vivo*. A. incorportated by selective pressure incorporation (SPI). B. incorporated by stop-codon suppression (SCS).

Up to date, 37 ncAAs (Figure 3) have been successfully incorporated into six lantibiotics *in vivo* (Table 1). The use of these approaches allows for the *in vivo* production of new lantibiotics with an expanded amino acid repertoire. By incorporating non-natural functional groups with novel and unique features, it dramatically expands the chemical and functional space of lantibiotic structures and enables the design of novel lantibiotic with enhanced properties (e.g. stability, specificity, bioavailability and half life).[40] For example, fluorinated ncAAs could be of interest to pharmaceutical industry since fluorination commonly improves the bioavailability and metabolic stability of drugs. In addition, ncAAs with reactive groups (e.g. alkyne or azide) can serve as chemical handles for click chemistry or other reactions to generate lantibiotic conjugates with fluorophores, glycans, PEGs, lipids, peptide moieties and other antimicrobial moieties.[43] With a general increase of chemical diversity, we intend to overcome the drawbacks that are usually found in peptide-based drugs.

2.2. Lantibiotics modification using click chemistry

Coupling moieties to lantibiotics and semisynthetic refinements of parent molecules offer exciting opportunities to produce novel lantibiotic derivatives with desirable properties enabling new functions and applications.[54] It has led to the development of lantibiotic derivatives with an increase in inhibitory activity against clinically relevant bacterial pathogens. The most prominent example is the C-terminal modification of deoxyactagardine B to yield NVB302, exhibiting improved activity and solubility compared to the parent molecule and this derivative has completed phase I clinical trials for the treatment of *C. difficile*.[10] A number of tools have been developed to achieve modifications of peptides and one of the most powerful and versatile synthetic tools is click chemistry. Click chemistry, referred to as "copper-catalyzed azide-alkyne cycloaddition (CuAAC)", was first reported by Sharpless and co-workers in 2001.[55] It is a region-selective copper (I) catalytic cycloaddition reaction between an azide and an alkyne that gives rise to a triazole. Due to its high level of reliability, specificity, biocompatibility, easiness to perform, and mild reaction conditions, click chemistry is being used increasingly in diverse areas, such as bioconjugation, drug discovery and polymer science.[56-58] Besides, the success of click chemistry for peptide modification can be attributed to the resulting triazole ring that can mimic an amide bond well and increases the stability and resistance to proteases by readily aligning with the biological targets through hydrogen bonding and dipole interactions.[59]

Lantibiotics modification using click chemistry has been the subject of several studies for the development of the target-specific bacterial probes and expanding its bioactivity and application. For example, to expand the activity spectrum of lantibiotic to Gram-negative bacteria, gallidermin has been conjugated with various siderophores.[60] All of the conjugates retained activity against the Gram-positive indicator strain. Even

though the penetration of the outer membrane was observed, they were unable to inhibit the growth of Gram-negative bacteria. The C-terminus of nisin has also been linked to fluorophores. The fluorescently labeled nisin was obtained retaining activity and it can now be used as molecular tool to increase the insight of the mechanistic details of the mode of action of nisin and other related lantibiotics.[61] Nisin's unique mode of action and potent activity make it an attractive candidate template for the development of new antibiotics. However, the proteolytic degradation and instability of the dehydroresidues limits the possible therapeutic application of the full-length peptide by oral delivery and injection. The lipid II-binding motif (rings AB) of nisin has been conjugated with various functional molecules. The combination of nisin AB with vancomycin increased the chelating efficiency of lipid II binding and drastically increased the activity of the conjugate against VRE.[62] The hybrid of the lipid II-binding motif of nisin and linear peptoids resulted in semisynthetic molecules that showed similar activity against MRSA.[63] The coupling of nisin AB to lipid moieties rendered semisynthetic hybrids with superior stability and potent antimicrobial activities against drug-susceptible and -resistant strains of Gram-positive bacteria including MRSA and VRE. The unique lipid II-mediated mode of action, its superior stability, and potent activity against pathogens of these nisin AB-lipopeptide hybrids make them attractive candidates for further optimization and development as novel antibiotics.[64] These studies highlight how lantibiotics can serve as lead structures to enhance their bioactivity and functional property via chemical coupling.

3. Outline of thesis

The research presented in this thesis mainly focus on lanthipeptides engineering. We utilized three differrent strategies to engineer lanthipeptides, therefore producing novel antimicrobials. These approaches were able to broaden the structure diversity of lanthipeptides, expanded our understanding of structure-activity relationship, and have also led to the development of lantibiotic derivatives with enhanced functionality in terms of activity spectrum, stability and specific activity against clinical relevant antibiotic-resistant pathogens. Additionally, this thesis also investigated the specificity and application of the lantibiotic protease NisP, which was proved to be a suitable protease for the activation of diverse heterologously expressed lantibiotics.

Chapter 1 provides a general introduction of lantipeptides, nisin, biosynthesis of nisin, the expression systems of lanthibiotic in *L. lactis*, engineering of lantibiotics by incorporation of ncAAs, and lantibiotics modification using click chemistry.

Chapter 2 investigates the specificity and application of the lantibiotic protease NisP. Two sets of nisin variants were constructed to test the ability of NisP to cleave leaders from various substrates. The first set was designed to study the influence of

variations in the leader peptide or variations around the cleavage site. The second set was designed to investigate the influence of the lanthionine ring topology. This study suggests that NisP has greater substrate tolerance than previously anticipated and it is the most suitable and inexpensive protease for the activation of diverse lantibiotics or thioether-stabilized peptides, produced with the nisin leader peptide and the modification machinery of nisin among all the proteases tested.

Chapter 3 describes the modular bioengineering of antimicrobial lanthipeptides aided by nanoFleming screening, a miniaturized and parallelized high-throughput inhibition assay developed by ETH collaborators. By combinatorial shuffling of 33 lantibiotic peptide modules derived from 12 antimicrobial lanthipeptides and 4 synthetic peptides, a library of 6,000 putatively active structures was generated. Screening of the library with the nanoFleming platform followed by characterization resulted in 11 antimicrobial lanthipeptides that showed enhanced antimicrobial activity compared to the wild-type peptides or were able to bypass resistance mechanisms.

In Chapter 4, we demonstrated for the first time the incorporation of methionine analogues into RiPPs in *L. lactis*. Four methionine analogues with unsaturated and varying side chain length were successfully incorporated at four different positions of nisin. The incorporation efficiency of ncAAs were analysed and declined in the order Aha > Hpg > Nle > Eth. The antimicrobial activities of 12 nisin derivatives were investigated. This study suggests that replacement of Met with Met analogues can alter the antimicrobial activity spectrum.

Chapter 5 presents two efficient and direct methods for the preparation of nisin conjugates via click chemistry. In the first method, C-terminally functionalized nisin AB and nisin ABC were conjugated to five hydrophobic pentynoyl peptides by Dr. Kubyshkin. The resulting semi-synthetic nisin analogues displayed potent inhibition of bacterial growth. In the second approach, nisin derivatives containing reactive groups (i.e. alkyne or azide) generated in work described in **Chapter 4** were utilized to conjugate nisin with peptide moieties and fluorescent probes. Six dimeric nisin constructs, three nisin hybrids and six fluorescently labeled nisin variants were prepared and their antimicrobial activities were retained, which substantiates the potential of this approach as a tool to study the localization and mode of action of nisin.

Lastly, the results described in this thesis and future perspectives are summarized and discussed in **Chapter 6**.

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CHAPTER 2

Specificity and application of the lantibiotic protease NisP

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Abstract

Lantibiotics are ribosomally produced and posttranslationally modified antimicrobial peptides containing several lanthionine residues. They exhibit substantial antimicrobial activity against Gram-positive bacteria, including relevant pathogens. The production of the model lantibiotic nisin minimally requires the expression of the modification and export machinery. The last step during nisin maturation is the cleavage of the leader peptide. This liberates the active compound and is catalyzed by the cell wall-anchored protease NisP. Here, we report the production and purification of a soluble variant of NisP. This has enabled us to study its specificity and test its suitability for biotechnological applications. The ability of soluble NisP to cleave leaders from various substrates was tested with two sets of nisin variants. The first set was designed to investigate the influence of amino acid variations in the leader peptide or variations around the cleavage site. The second set was designed to study the influence of the lanthionine ring topology on the proteolytic efficiency. We show that the substrate promiscuity is higher than has previously been suggested. Our results demonstrate the importance of the arginine residue at the end of the leader peptide and the importance of lanthionine rings in the substrate for specific cleavage. Collectively, these data indicate that NisP is a suitable protease for the activation of diverse heterologously expressed lantibiotics.

Introduction

Lanthipeptides are posttranslationally modified peptides that contain dehydrated amino acids and (methyl) lanthionine residues.[1,2] Lantibiotics are those lanthipeptides that have significant antimicrobial activity, mostly found within classes I and II. Some lantibiotics show activity against clinically relevant bacteria in a concentration range comparable to antibiotics in use. Moreover, they can target multidrug resistant bacteria.[3,4] The production of the model lantibiotic nisin (belonging to the class I lanthipeptides) by *Lactococcus lactis* requires the coordinated expression of 11 genes.[5] Precursor nisin is produced as a linear precursor peptide that undergoes dehydration and cyclization in a directional and alternating way[6] and is subsequently exported by a dedicated transporter (NisT). Outside the cell, the protease NisP cleaves off the leader peptide releasing mature nisin.[7] In this process, the leader peptide of nisin serves as a recognition motif for the modification enzymes and the transporter and keeps the fully modified prenisin inactive until it is removed.[7–17]

The proteases involved in the maturation of lanthipeptides recognize different cleavage sites. The type I lanthipeptide proteases, generally referred to as LanP, are subtilisin-like serine proteases. They can be secreted to the extracellular medium, like EpiP[18], or remain in the cytoplasm, like PepP [19], or be exported and bound to the cell wall, like NisP. In the maturation of subtilin, no specific protease has been found and the processing takes place outside the cell probably by diverse serine proteases [20]. The first lantibiotic protease with a resolved 3D structure, EpiP from *Staphylococcus aureus*, an analogue of NisP, has been reported.[21,22] On the other hand, in type II lanthipeptides, the protease domain is fused to the transporter and this protein cleaves behind a double glycine motif.[2] In type III lanthipeptides, the cleavage is not so specific and is mediated by a prolyl oligopeptidase.[23]

The modification enzymes of the nisin biosynthesis gene cluster have been used to produce potent and stable variants of clinically relevant peptides, providing extensive information regarding the promiscuity of the modification machinery (NisBC) and the transporter (NisT).[24–32] The production of modified prelantibiotics allows to obviate the requirement for immunity and can achieve higher yields, although it requires a later activation by cleavage of the leader peptide.[33–35] Moreover, NisRK expressed in diverse strains provides a widely used inducible protein expression system for Gram-positive bacteria.[36,37]

The promiscuity of NisBTC and the development of an efficient production system for the modification and export of modified peptides enabled the production of putative lantibiotics from diverse bacteria in *L. lactis*.[32,33] Moreover, this production system can be extended with additional enzyme modules (i.e. additional modification enzymes found in lantibiotic gene clusters)[38] or with non-canonical amino acids[39–41]

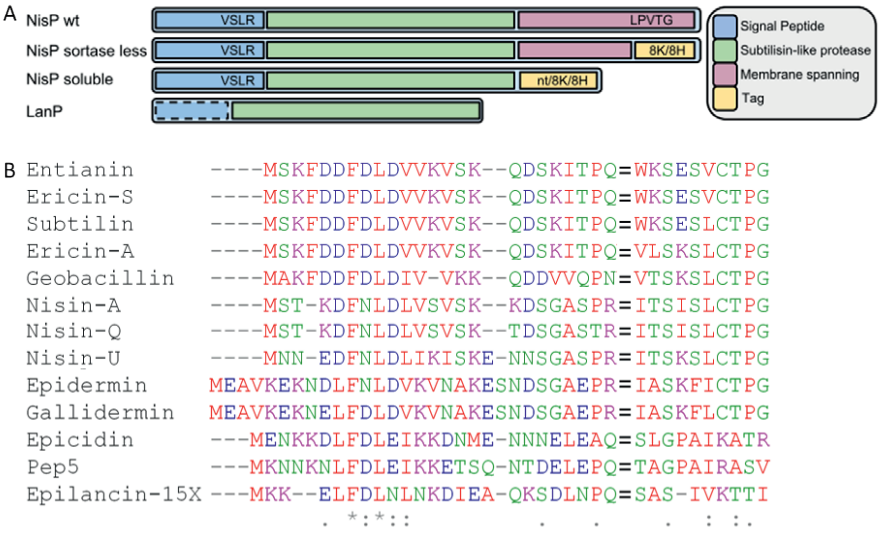

Figure 1. (**A**) Schematic view of wild-type NisP, NisP mutants generated in this work and other LanPs. 8H represents the 8-histidine tag, 8K represents the 8-lysine tag, and nt denotes no tag added. The dotted line around the signal peptide in LanP indicates that this part is not present in all LanP proteases. (**B**) Alignment of diverse type I lantibiotic peptides. The F(D/N)LD motif and the Pro-2 are highly conserved. The cleavage site is indicated with an equal to sign (=).

that increase the repertoire of unusual amino acids that can be incorporated *in vivo* in peptides. These findings highlight the large potential of using Synthetic Biology principles in the production of novel lanthipeptides.[34,42] The versatility of the NisBTC system allows for the production of a large number of putative prelantibiotics. Thus, a protease capable of releasing the active lantibiotic during growth of the producer strain is indispensable for using high-throughput screening systems on novel peptides. Additionally, the production of modified prelantibiotics can achieve higher yields because no immunity is required being the only drawback the necessity of a suitable leader peptidase.[35] Therefore, we tested the suitability of diverse commercial proteases for the cleavage of the leader peptide of nisin after maturation in various growth conditions. We compared their activity with that of the lantibiotic protease NisP, especially in view of the importance of the variable residues present behind the cleavage site, to establish the potential of NisP for various biotechnological applications.

The nisin protease, NisP, is produced and exported to the outside of the cell, where it is anchored to the peptidoglycan via a sortase-catalyzed coupling and performs its function, although a fraction of the protease escapes anchoring.[16,21,43] It contains a typical N-terminal secretion signal, a protease domain, a self-cleavage C-terminal sequence and a sortase recognition sequence (Figure 1A). The maturation of NisP involves the release of the signal peptide and part of the prepeptide, likely by self-cleavage of

the N-terminal sequence[16], as has been shown for an EpiP-analogue.[22] Although the role of NisP in the production of nisin is crucial, little is known about the specificity of the protease or the recognition sites present in prenisin that allow for the binding and cleavage of the leader peptide, although recently the influence of lanthionine rings on processing specificity has been reported.[44] Moreover, the fact that NisP is attached to the cell wall has prevented a detailed study of the specificity of NisP. Here, we present a systematic study of an engineered soluble variant of the lantibiotic protease NisP. This work will greatly facilitate the efficient production and activation of a wide variety of active lantibiotics with a cost-effectively produced protease.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and vectors used in this work are listed in Table 1. Lactococcal strains were grown in M17 (Oxoid), supplemented with 0.5% glucose (GM-17) at 30 °C for genetic manipulation or in the same conditions, but in MEM for peptide production.[31] *Escherichia coli* and *Micrococcus flavus* strains were grown in LB at 37 °C, while shaking at 250 rpm. When appropriate, erythromycin and/or chloramphenicol (Sigma-Aldrich) were added at a final concentration of 5 µg/mL. Kanamycin (Sigma-Aldrich) was used at a final concentration of 20 µg/mL.

Construction of expression vectors.

Cloning steps were performed following standard protocols.[45] The preparation of competent *L. lactis* cells and transformation were carried out according to Holo and Nes.[46] Restriction endonucleases and ligase were used as recommended by the provider (Thermo Scientific).

For the cloning of *nisP* variants, the gene was amplified from the genome of *L. lactis* NZ9700 using primers nisPbsphfwd and nisP8KXbarev or nisPbsphfwd and nisP8KSacIr for the addition of a 8-mer poly-lysine tag, nisPbsphfwd and nisP8HXbarev or nisPbsphfwd and nisP8HSacIr for the addition of a 8-mer poly-histidine tag, or nisPbsphfwd and solNisPcontrol for the production of an untagged soluble NisP (Supplementary Table 1). The amplification was performed using Phusion Polymerase (Thermo Scientific) following the provider's instructions. After amplification, the DNA was purified using the PCR cleaning kit (Roche) and digested with *Bsp*HI and *Xba*I or *Bsp*HI and *Sac*I and ligated in pNZ8048 digested with *Nco*I and either *Xba*I or *Sac*I. Similarly, the fragment was inserted into pET28b digested with *Nco*I and either *Spe*I or *Sac*I. The ligation mix was transformed into *L. lactis* NZ9000 or *E. coli* Rossetta Blue DE3. The nucleotide sequence of each gene was checked by sequencing with the primers listed in table 1.

Table 1. Strains and vectors used in this work.

Strain	**Characteristics**	**References**
Lactococcus lactis NZ9000	*nisRK::pepN*	47
Lactococcus lactis NZ9700	Nisin producer	7
Micrococcus flavus B423	Sensitive strain	NIZO Food Research
Escherichia coli Rossetta Blue DE3	Expression host	Novagen
Plasmid	**Characteristics**	**References**
pET28b	Vector with the IPTG inducible PT7. KmR	Novagen
pNZ8048	CmR PnisA	[36]
pNZE3-empty	EryR	[38]
pIL3BTC	CmR PnisA-*nisBTC*	[31]
pNZnisA-E3	EryR PnisA-nisA	[27]
pNZE3-Cys-less	C-terminal His-tagged Nisin (C7A C11A C18A C25A C27A) mutant. EryR	[11]
pIL253	EryR	[48]
pNGnisTP	CmR PnisA-*nisTP*	[27]
pNZnisP-8H	PnisA-*nisP* with 8 histidines tag after the subtilisin-like domain. CmR	This work
pNZnisP-8K	PnisA-*nisP* with 8 lysines tag after the subtilisin-like domain. CmR	This work
pNZnisP-sol	PnisA-*nisP* with no tag fused after the subtilisin-like domain. CmR	This work
pNZnisPsl-8H	PnisA-*nisP* sortase-less with 8 histidines tag. CmR	This work
pNZnisPsl-8K	PnisA-*nisP* sortase-less with 8 lysines tag. CmR	This work
pETNisP-sol	PT7-*nisP* with no tag fused after the subtilisin-like domain. KmR	This work
pETNisP-8H	PT7-*nisP* with 8 lysines tag after the subtilisin-like domain. KmR	This work
pETnisP-8K	PT7-*nisP* with 8 lysines tag fused after the subtilisin-like domain. KmR	This work
pNZE3nisA-C7A-ASPR	Nisin C7A mutant. EryR	This work
pNZE3nisA-Cysless-ASPR	Nisin (C7A C11A C18A C25A C27A) mutant lacking all cysteines. EryR	This work
pNZE3nisA-CAAAA-ASPR	Nisin (C11A C18A C25A C27A) mutant retaining only the first cysteine in the prepeptide. EryR	This work
pNZE3nis-ringAless-ASPR	Nisin (T2V S3A S5A C7A) mutant, EryR	This work
pNZE3nisA-VSLR	Nisin (A-4V P-2L) mutant containing a VSLR instead of the typical ASPR sequence in the leader, EryR	This work
pNZE3nisA-C7A-VSLR	Nisin (A-4V P-2L C7A) mutant with a VSLR NisP cleavage site, EryR	This work
pNZE3nisA-Cysless-VSLR	Nisin (A-4V P-2L C7A C11A C18A C25A C27A) mutant lacking all cysteines and with a VSLR NisP site, EryR	This work
pNZE3nisA-CAAAA-VSLR	Nisin (A-4V P-2L C11A C18A C25A C27A) mutant retaining only the first cysteine in the prepeptide and with a VSLR NisP site, EryR	This work
pNZE3nisA-ringAless-VSLR	Nisin (A-4V P-2L T2V S3A S5A C7A) mutant with a VSLR NisP site, EryR	This work

Strain	Characteristics	References
pNZE3nisA-I1D	Nisin I1D mutant, EryR	This work
pNZE3nisA-I1W	Nisin I1W mutant, EryR	This work
pNZE3nisA-I1K	Nisin I1K mutant, EryR	This work
pNZE3nisA-T2K	Nisin T2K mutant, EryR	This work
pNZE3nisA-T2V	Nisin T2V mutant, EryR	This work
pNZE3-DDDK	NisP cleavage site ASPR replaced by DDDK, EryR	[14]
pNZE3-DDDDK	NisP cleavage site GASPR replaced by DDDDK, EryR	[14]
pNZE3-AFNLD	Nisin D-19A mutant, EryR	[14]
pNZE3-DANLD	Nisin F-18A mutant, EryR	[14]
pNZE3-DFALD	Nisin N-17A mutant, EryR	[14]
pNZE3-DFNAD	Nisin L-16A mutant, EryR	[14]
pNZE3-DFNLA	Nisin D-15A mutant, EryR	[14]
pNZE3-nis-V8	Nisin Z R-1E mutant, EryR	This work
pNZE3-nis-Fx	Nisin Z (A-4I S-3E P-2G) mutant with IEGR replacing the ASPR NisP cleavage site, EryR	This work
pNZE3-nis-Thr	Nisin Z (S-3V) mutant with AVPR replacing the ASPR NisP cleavage site, EryR	This work
pNZE-nisΔ(23-34)	Nisin Δ(23-34) deletion mutant, EryR	[30]
pNZE3-1765	nisin leader peptide fused to the leaderless part encoded by spr1765, EryR	[33]
pNZE3-1766	nisin leader peptide fused to the leaderless part encoded by spr1766, EryR	[33]

CmR: chloramphenicol resistance, EryR: erythromycin resistance.

For the construction of pNZE3nisA-CAAAA-ASPR, the nisin CAAAA coding gene (last 4 Cys replaced by Ala) was synthesized by GeneArt and cloned into pNZE3-empty[38] as a *Bgl*II-*Hind*III fragment encoding the P*nisA* promoter and *nisin-CAAAA*. For the construction of pNZE3nisA-Cysless-ASPR, Nisin Cys-less was amplified from pNZE3-Cys-less[11] using the primers pNZE3Emf and C-lessH6-less, digested with *Bgl*II and *Hind*III, and cloned into pNZE3-empty cut with the same enzymes. pNZE3nisA-C7A-VSLR was produced by round PCR of pNZnisA-E3 using the primers NisPC7A-rev and NisPC7A-fwd. pNZE3nisA-C7A-ASPR was produced by round PCR of pNZE3nisA-C7A-VSLR using the primers NisPC7A-rev and NisPC7A-ASPR-fwd. The equivalent genes in which the end of the leader peptide was mutated from ASPR to VSLR were generated by round-PCR of each of the plasmids mentioned above with the primers nisVSLRfwd and nisVSLRrev.

The nisin variants with a mutation in the first two amino acids of the core peptide were produced by round PCR of pNZnisA-E3 using phosphorylated P-for as a forward primer in all cases and P-IK-Rev, P-KT-Rev, P-WT-Rev, P-DT-Rev or P-IV-Rev for Nisin A-T2K, Nisin A-I1K, Nisin A-I1W, Nisin A-I1D and Nisin A-T2V mutants, respectively.

Gene expression and product purification.

An overnight culture of *L. lactis* NZ9000 grown in GM17 with the desired plasmid(s) was diluted 50-times in preheated MEM and grown until an OD 600 nm of 0.4–0.6. At this moment, the culture was induced with 5 ng/mL of nisin (Sigma-Aldrich). Cells were harvested after 3 h of induction and the supernatants containing the protein of interest were further purified.

Trichloroacetic acid (TCA) precipitation was carried out according to Sambrook et al.[45] The purification of nisin and its mutants in higher amounts was performed according to described protocols.[6] When higher purity was required, the fractions collected were applied to a spherical C18 versaflash column (Supelco) previously equilibrated in 0.1% trifluoroacetic acid (TFA). The column was washed in three steps with 3 volumes of 33%, 66% and 100% organic solvent (2:1 isopropanol:acetonitrile 0.1% TFA). After this step, the peptides were concentrated by freeze-drying.

For the purification of soluble truncated NisP, the producer cells were grown and induced at an OD 600 nm of 0.4–0.6 with either 5 ng/mL nisin or 1 mM IPTG depending on the producer strain being *L. lactis* NZ9000 or *E. coli* Rossetta Blue DE3, respectively. The cells were separated after 3 h induction by centrifugation at 6000 rpm for 10 min at 4 °C. The his-tagged variant NisP-8H was purified by affinity chromatography using a Ni-NTA fast flow resin (Qiagen). Briefly, the cell-free supernatant of *L. lactis* strains or the cell-lysate of *E. coli* strains was passed through a column previously equilibrated with binding buffer (20 mM phosphate buffer 0.5 M NaCl pH 8.0). The column was washed with 50 mM phosphate buffer 0.5 M NaCl 20 mM imidazole pH 8.0. NisP-8H was eluted from the column using 50 mM phosphate buffer 0.5 M NaCl 250 mM imidazole. NisP-8K was purified by cationic exchange chromatography using a Fast-flow SP-sepharose (GE Healthcare). The column was equilibrated with 5 column volumes of 20 mM phosphate buffer pH 6.5. The pH of the supernatant was adjusted to pH 6.5 and then passed through the column. After washing with 5 column volumes of 20 mM phosphate pH 6.5 0.5 M NaCl, the attached protein was eluted with 20 mM phosphate pH 6.5 1.5 M NaCl.

The presence of NisP and its variants in elution fractions was assessed by checking the ability to activate prenisin in antimicrobial assays and/or by SDS-PAGE according to Laemmli.[49]

Proteolysis of nisin and nisin mutants using NisP

Lyophilized nisin or its mutants were solubilized using a 0.05% acetic acid solution. Different buffers were prepared: 1 M HEPES, 1 M NaCl, 1 M HEPES 50 mM $CaCl_2$, 1 M HEPES 50 mM $MgCl_2$, 1 M MES, 1 M MES 50 mM $CaCl_2$. In all cases, the pH was adjusted to 6.5 and mixed with each sample and then diluted 10 times. Additionally, 1 M Tris 50 mM $CaCl_2$ pH 6.0 was tested. The pH was measured after mixing and corrected if necessary.

Alternatively, supernatants containing nisin mutant peptides produced after induction were divided into two fractions; one in which the pH was adjusted to 6.0 and the other one where the pH was the actual fermentation pH. They were incubated overnight at 37 °C with or without His-tagged purified NisP at a ratio of 1000:1. After the incubation, the supernatants were precipitated using TCA and resuspended in 1/20 vol 0.05% acetic acid. For the mutants that were produced in lower amounts, larger volumes were induced and the peptides were purified by cationic exchange chromatography as described before and separated by reverse phase chromatography using a Jupiter 4 µm Proteo 90 Å 250 × 4.6 mm C12 analytical column (Phenomenex). The column was equilibrated in 20% organic solvent (acetonitrile 0.1% TFA) before the sample injection. It was washed for 5 min before applying a linear gradient from 20% to 50% organic solvent in 20 min. 1 µl of each collected peak was used for mass-spectrometric determination.

Mass spectrometry

Mass spectrometry analysis of the samples was performed in an ABI Voyager DE Pro (Applied Biosystems) operating in linear mode as previously described using external calibration (van Heel et al., 2013). Briefly, 1 µL sample was spotted, dried and washed with 5 µL Milli-Q water, on the target. Next, 1 µL of α-cyano-4-hydroxycinnamic acid 5 mg/mL (Sigma-Aldrich) was spotted on the sample.

Activity test

Activity tests were performed by well-diffusion assay as indicated by van Heel et al.[38] The cleavage of nisin by NisP after SDS-PAGE was monitored washing the gel according to Bhunia et al. [50] and covering with an overlay of GM17 soft agar inoculated with NZ9000 (pIL3BTC pNZnisA-E3) induced with 1 ng/mL nisin. The overlay was incubated for 16 h, after which the presence of inhibition zones was evaluated.

Enzyme kinetic assays

In order to investigate the substrate specificity and kinetic parameters of NisP, a set of mutants of P4-P1 was created. We used wild-type prenisin (ASPR), nis-Peng (VSLR), nis-Thrombin (AVPR), and nis-Factor Xa (IEGR) as substrates. The concentration of NisP was determined by the BCA assay with bovine serum albumin as standard. The conditions of cleavage reaction were optimized with 100 mM Tris buffer containing 5 mM CaCl2 (pH 6.0). The reaction was stopped at the indicated times adding TFA to a final concentration of 1%. The reaction was performed in 100 µl with 6.5 ng/mL NisP at 37 °C. 1% TFA was added to terminate the reaction at 5 different time points (5 min, 15 min, 30 min, 45 min, and 60 min). All the samples were analyzed by analytical RP-HPLC as indicated before[51] and measuring the absorbance at 205 nm. For each

substrate concentration, the initial velocity was calculated on the basis of the peak area of released nisin versus time. The kinetic parameters were determined by fitting the calculated enzyme activities at different substrate concentrations (ranging from 1 to 25 μM) to a linear regression curve on Lineweaver–Burk double reciprocal plot.[52]

Results

Cloning and expression of a soluble NisP variant

The fact that NisP is a cell wall anchored protease has hindered a thorough characterization and assessment of its biotechnological properties. In a first attempt to produce soluble NisP, we designed primers that hybridize partially to the sequence of NisP immediately upstream of the sortase recognition sequence in the C-terminus of NisP (LPVTG). We designed the primers nisP8KXbarev and NisP8HXbarev, which add a tail of 8-Lys or 8-His residues, respectively. These tags facilitate the purification of the protease and can also serve for the immobilization on different materials for high-throughput applications.

The supernatants of the strains NZ9000 (pNZnisPsl-8H) and NZ9000 (pNZnisPsl-8K) were tested for the presence of protease activity after 3 h of induction with nisin. In each case, 5μl of the supernatant was mixed with 50 μl of prenisin obtained by TCA precipitation of induced NZ9000 (pIL3BTC pNZnisA-E3). Positive controls using trypsin or the supernatant of induced NZ9000 (pNGnisTP) and a negative control containing only prenisin were used. The presence of an appropriate protease in the mix will release active nisin, thus we can monitor the activity of NisP by measuring the antimicrobial activity against the sensitive strain *Micrococcus flavus*. We could observe antimicrobial activity only in the positive controls, thus indicating that the variant NisP protease, if produced, is not active (data not shown).

In a second approach, we attempted to reduce the size of NisP, while keeping the active subtilisin-like domain intact. An alignment comparing different LanP proteases shows that NisP has a hydrophobic helix after the protease domain which is not present in other intracellular proteases.[53] Thus, we decided to express a NisP variant that lacks the sortase recognition sequence and this motif. Similarly, we amplified the gene adding a C-terminal tag of either 8 histidines, or 8 lysines or no tag. We repeated the activity test with the supernatants of induced *L. lactis* NZ9000 (pNZnisP-sol), NZ9000 (pNZnisP-8H) and NZ9000 (pNZnisP-8K) (Figure 2A). In this case we were able to detect antimicrobial activity for all the soluble truncated NisP variants, with or without a tag attached to the C-terminus of the protein. Additionally, the size of the inhibition halo measured is larger in the samples activated with the soluble protease present in the supernatants of these three engineered strains compared to the samples activated with the supernantant of *L. lactis* NZ9000 (pNGnisTP), which produces as a major

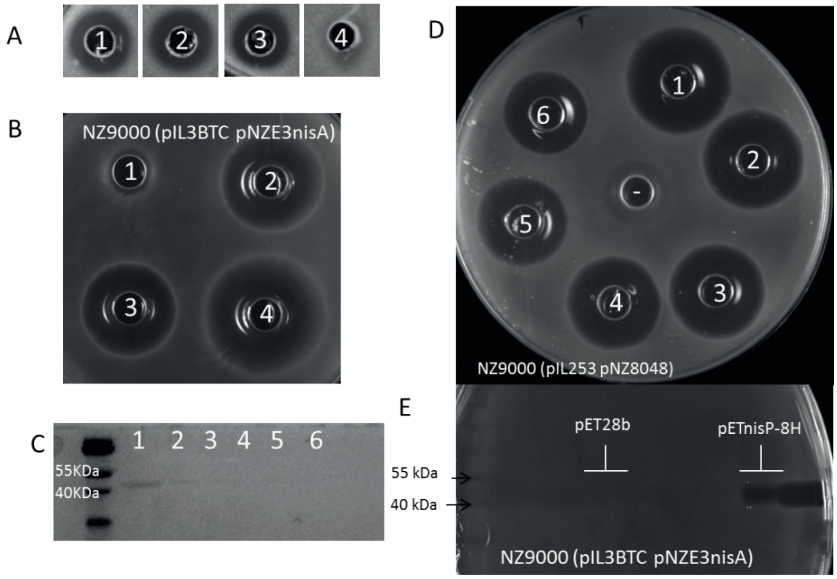

Figure 2. Activity of engineered NisP variants. **A)** Activation of prenisin with supernatants of induced *L. lactis* NZ9000 transformed with pNZnisP-sol (1), pNZnisP-8H (2), pNZnisP-8K (3), or pNGnisTP (4), using *M. flavus* as an indicator strain. **B)** Activity of NisP heterologously produced in *E. coli* Rossetta Blue DE3 using the prenisin producing strain *L. lactis* NZ9000 (pNZnisA-E3 pIL3BTC) both as producer and as sensitive strain. 20 µl of cell lysate of *E. coli* (pET28b) (1), *E. coli* (pETNisP-sol) (2) or *E. coli* (pETNisP-8H) (3) were added to the wells. 1 µl of NisP-8H purified from *L. lactis* NZ9000 (pNZnisP-8H) was used as a positive control (4). **C)** SDS-PAGE of NisP-8H purified by affinity chromatography from *L. lactis* NZ9000 (pNZnisP-8H). Wells 1-6 contain 15 µl of each 2 mL-fraction collected. **D)** Activation of prenisin by NisP-8H purified from *L. lactis* NZ9000 (pNZnisP-8H) using *L. lactis* NZ9000 (pNZ8048 pIL253) as a sensitive strain. Wells 1-6 correspond to a mix of 50 µl of supernatants of induced *L. lactis* NZ9000 (pNZnisA-E3 pIL3BTC) and 2 µl of purified NisP from the batch shown in panel C. **E)** Activity test of NisP-8H from *E. coli* Rossetta Blue DE3 (pETnisP-8H) after separation by SDS-PAGE. *L. lactis* NZ9000 (pNZnisA-E3 pIL3BTC) was used both as producer and as sensitive strain.

product wild-type cell-anchored NisP. This indicates that the amount of truncated NisP in the supernatants is higher than the amount of wild-type NisP exposed by the cells. These results demonstrate that the engineered NisP variants containing only the protease domain can be recovered from the supernatants. It also shows that C-terminally tagged NisP retains good activity.

The truncated NisP variants were also cloned in an *E. coli* expression vector, rendering pET28-NisP-8H, pET28-NisP-8K and pET28-NisP-sol. The cell pellet obtained after induction of *E. coli* Rosetta Blue DE3 transformed with these vectors was disrupted using glass beads and used in an antimicrobial assay to activate prenisin. It was possible to activate prenisin using this fraction, showing the ability of *E. coli* to produce active truncated NisP (Figure 2B). Moreover, the supernatants of the induced cultures were also able to activate prenisin whereas the control did not.

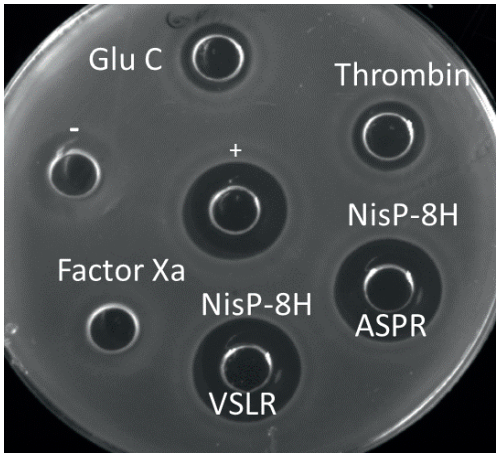

Figure 3. Activity of the proteases in crude supernatants. Antimicrobial assay against *L. lactis* NZ9000 (pIL253 pNZ8048) using different proteases. Each protease (1 µl) was mixed with supernatant containing 50 µl of its corresponding prenisin variant. Activity observed results from protease activity on the substrate releasing active nisin. + indicates a positive control of 50 µl nisin 10 ng/µl. – indicates a negative control of untreated prenisin. Supernatants of induced *L. lactis* NZ9000 (pIL3BTC pNZE3nis-V8) (Glu C), NZ9000 (pIL3BTC pNZE3nis-Thr) (Thrombin), NZ9000 (pIL3BTC pNZE3nisA-ASPR) (NisP-8H ASPR), NZ9000 (pIL3BTC pNZE3nisA-VSLR) (NisP-8H VSLR), NZ9000 (pIL3BTC pNZE3nis-Fx) (Factor Xa) were mixed with 1 µl of the specific protease.

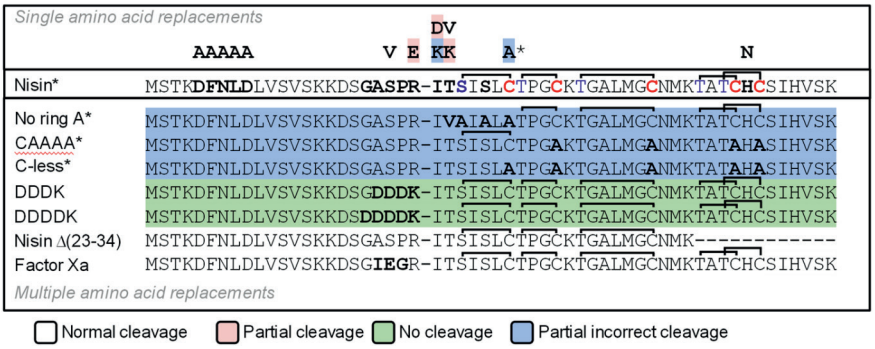

Figure 4. Schematic representation of the cleavage of nisin mutants using NisP-8H. Single mutants are indicated with one letter above wild-type nisin sequence. Mutants with several amino acid replacements are depicted in bold letters under wild-type nisin sequence. Lanthione rings are represented as a continuous line connecting Ser or Thr to Cys. The * indicates that this result was also obtained using the same variant with a VSLR cleavages site instead of the wild type ASPR site.

Purification of NisP and optimization of cleavage conditions

The strains *L. lactis* NZ9000 (pNZnisP-8H) and NZ9000 (pNZnisP-8K) were induced and the production of protease was monitored by SDS-PAGE of the diverse fractions collected during the purification by either affinity chromatography or cation exchange

chromatography, respectively. We could clearly detect a highly pure band of approximately 42 kDa, which is the expected size after removal of the signal peptide during production (Figure 2C) with an average yield of 1 mg/L. The protease activity of each fraction was monitored by activation of prenisin (Figure 2D). Surprisingly, even in fractions for which the sensitivity of Coomassie blue staining was too low to detect the protease, activity could still be detected. Similarly, after induction of *E. coli* Rosetta Blue DE3 (pET28-NisP-8H) or (pET-NisP-8K) a protein of approximately 42 kDa was detected in the supernatants by SDS-PAGE. The gel was covered with a prenisin producing *L. lactis* strain demonstrating that this protein could activate prenisin and create an inhibition zone (Figure 2E).

The activity of NisP on wild-type prenisin was tested in different buffers based on Tris, HEPES or MES with or without calcium and magnesium and monitored by HPLC. We conducted the experiments in duplicate at 30 and 37 °C. We found that the cleavage in 100 mM tris buffer pH 6.0 supplemented with 5 mM $CaCl_2$ at 37 °C was the optimal buffer for the reaction, although the protease was still reasonably active in the other conditions (data not shown).

Prenisin specific cleavage using specific proteases in culture conditions

To further compare the versatility of NisP in culture conditions with various proteases frequently used in biotechnology, we mutated the last four amino acids in the leader peptide (P4-P1) of nisin (i.e. ASPR) to insert a factor Xa (IEGR) or a thrombin (AVPR) cleavage site. We also mutated the arginine in position P1 of the leader peptide into glutamic acid, creating a cleavage site for the endoprotease Glu-C. Additionally, the proposed self-cleavage sequence of NisP (VSLR) (Figure 1 (van der Meer et al., 1993)) was used to replace the ASPR sequence at the end of the nisin leader peptide. The supernatants of the prenisin producer strains containing these mutations were placed in a well on the agar plate and the specific proteases were added. The cleavage was evaluated by activity against NZ9000 (pIL253 pNZ8048), which is resistant to the erythromycin and chloramphenicol present in the supernatants of the substrate producer strains but sensitive to nisin. In these conditions, except for Factor Xa, all the proteases tested (thrombin, NisP and endoprotease Glu-C) were active (Figure 3).

Specificity of NisP

We designed a set of nisin mutants to investigate the specificity of NisP (Figure 4). For all these mutants, supernatants were collected after induction and divided into three fractions. NisP was added to two of these fractions, one maintaining the pH of the culture (4.5-5.0) and the other fraction was adjusted to pH 6.0. The third fraction was used as an untreated control. After cleavage, the mass of the peptides were monitored by MALDI-TOF (Table 2).

Table 2. Cleavage of nisin mutants using NisP on crude supernatants of the producer strain directly after fermentation or with the pH adjusted to 6.0.

L. lactis NZ9000 (pIL3BTC)	Expected		Measured			Dehydrations
	-NisP	+NisP	-NisP	+NisP	+NisP pH 6.0	
pNZnisA-E3	5686.4	3354.2	5689.9 (+4.5)	3357.8 (+3.6)	3353.8 (-0.4)	8
		3372.2		3372.8 (+0.6)	3370.3 (-1.9)	7
		3390.2		3388.0 (-2.2)	3387.9 (-2.2)	6
pNZnisAC7A-ASPR	5654.3	3322.1	5663.9 (+9.6)	3322.7 (+0.6)		8
		3240.1		3340.0 (-0.1)		7
		3358.1		3357.3 (-0.8)		6
		3376.1		3374.9 (-1,2)		5
		3476.7		3478.8 (+2.1)		8 (+R)
		3494.7		3493.8 (-0.9)		7 (+R)
		3512.7		3512.2 (-0.5)		6 (+R)
		3208.9			3208.8 (-0.1)	8 (-I)
		3226.9			3225.5 (-1.4)	7 (-I)
		3244.9			3241.9 (-3.0)	6 (-I)
pNZE3nisA-ringAless-ASPR	5669.3	3337.1	5679.3 (+10.0)	3338.8 (+1.7)	3338.8 (+1.7)	4
		3355.1		3357.9 (+2.8)	3357.1 (+2.0)	3
		3142.8		3141.2 (-0.6)	3142.0 (-0.8)	3-IV
pNZE3nisA-CAAAA-ASPR	5540.1	3207.9		3207.8 (-0.1)	3210.3 (+2.4)	9
	5558.1	3225.9	5556.9 (-1.2)	3224.1 (-1.8)	3227.3 (+1.4)	8
		3243.9		3239.9 (-4.0)	3241.6 (-2.3)	7
pNZE3nisA-Cysless-ASPR	5526.1	3175.9	5528.0 (+1.9)	3175.9 (0)	3175.2 (-0.7)	9
		3193.9		3191.7 (-2.2)	3189.9 (-4.0)	8
		3332.1			3330.9 (-1.2)	9 (+R)
pNZE3nisA-VSLR	5730.4	3354.2	5728.6 (-1.8)	3353.0 (-1.2)	3353.7 (-0.5)	8
		3372.2		3368.9 (-3.3)	3366.8 (-5.4)	7
		3390.2		3385.9 (-4.3)	3390.9 (+0.7)	6
pNZE3nisA-C7A-VSLR	5716.4	3322.1	5722.6 (+6.2)	3322.1 (0)	3322.9 (+0.8)	8
		3340.1		3337.2 (-2.9)		7
		3478.3		3478.3 (0)	3479.1 (+0.8)	8 (+R)
		3496.3		3493.9 (-2.4)	3495.2 (-1.1)	7 (+R)
		3591.4		3590.2 (-1.2)		8 (+LR)
		3609.4		3608.6 (-0.8)		7 (+LR)

L. lactis NZ9000 (pIL3BTC)	Expected		Measured			Dehydrations
	-NisP	+NisP	-NisP	+NisP	+NisP pH 6.0	
pNZE3nisA-ringAless-VSLR	5711.9	3337.1	5717.9 (+6.0)	3333.5 (-3.6)	3343.9 (+6.8)	4
		3223.9		3231.1 (+7.2)	3230.8 (+6.9)	4 (-I)
pNZE3nisA-CAAAA-VSLR	5602.2	3207.9	5596.2 (-6.0)	3207.3 (-0.6)	3207.8 (-0.1)	8
		3225.9		3223.4 (-2.5)	3223.9 (0)	7
		3243.9			3239.9 (-4.0)	6
pNZE3nisA-Cysless-VSLR	5552.1	3175.9		3177.8 (+1.9)	3166.7 (-9.2)	9
	5588.1		5583.6 (-4.5)			7
		3366.8		3361.1 (-5.7)	3365.5 (-1.3)	7 (+R)
		3445.2			3444.2 (-1.3)	8 (+VR)
		3649.4		3650.9 (+1.5)	3655.5 (+6.1)	7 (+VSLR)
pNZE3nisA-I1K	5737.4	3369.2	5741.6 (+4.2)	3369.9 (+0.7)	3369.6 (+0.4)	6
	5755.4	3387.2	5764.7 (+9.3)	3387.6 (+0.4)	3385.9 (-1.3)	5
		3405.2		3404.8 (-0.4)	3403.6 (-1.6)	4
		3525.4		3522.1 (-3.3)		8 (+R)
pNZE3nisA-T2K	5729.9	3373.2			3373.0 (-0.2)	7
	5747.9	3391.2	5746.2 (-1.7)	3396.4 (+5.2)	3391.0 (-0.2)	6
pNZE3-nisA-T2V	5702.4	3370.2		3370.1 (-0.1)	3770.0 (-0.2)	7
	5720.4	3388.2		3387.3 (-0.9)	3388.3 (+0.1)	6
	5738.4	3406.2		3404.5 (-1.7)	3405.6 (-0.6)	5
	5756.4		5756.4 (0)			4
	5774.4		5781.7 (+7.3)			3
pNZE3-nisA-I1D	5706.3	3374.1		3373.4 (-0.7)	3373.7 (-0.4)	7
	5724.3	3392.1		3391.2 (-0.9)	3391.7 (-0.4)	6
	5742.3	3410.1	5746.6 (+4.3)		3407.7 (-2.4)	5
pNZE3-nisA-I1W	5803.4	3427.1	5808.5 (+4.1)	3425.8 (-1.3)	3424.7 (+0.6)	8
	5821.4	3445.1	5820.9 (-0.5)	3444.1 (-1.4)	3443.9 (-1.2)	7
	5839.4	3463.1		3460.9 (-2.2)	3464.2 (+1.1)	6
	5857.4		5855.3 (-2.1)			5
pNZE3-nisA-V8	5636.3	3331.1	5646.4 (+10.1)	3331.3 (+0.2)	3334.3 (+3.2)	8
		3349.1		3348.0 (-1.1)	3353.4 (+4.3)	7
pNZE3-nisA-Thr	5675.4	3331.1	5685.4 (+10.0)	3330.5 (-0.6)	3326.9 (-4.2)	8
		3349.1		3350.0 (+0.9)	3350.1 (+1.0)	7
pNZE3-nisA-FX	5707.4	3331.1	5709.5 (+2.1)	3334.7 (+3.6)	3332.9 (+1.8)	8
	5725.4		5727.6 (+2.2)			7

L. lactis NZ9000 (pIL3BTC)	Expected		Measured			Dehydrations
	-NisP	+NisP	-NisP	+NisP	+NisP pH 6.0	
pNZE3nisA-DDDK	5748.3	3354.2	5744.9 (-3.4)		5743.2 (-5.1)	8
	5766.3			5763.2 (-3.1)		7
pNZE3-DDDDK	5806.3	3354.2	5804.1 (-2.2)	5812.3 (+6.0)		8
	5824.3				5824.6 (+0.3)	7
pNZE3-AFNLD	5642.4	3354.2	5643.4 (+1.0)	3355.1 (+0.9)		8
		3372.2		3378.8 (+6.6)	3371.8 (-0.4)	7
pNZE3-DANLD	5628.3	3354.2	5629.9 (+1.6)	3353.0 (-1.2)	3350.7 (-3.5)	8
		3372.2		3370.1 (-2.1)	3367.8 (-4.4)	7
		3390.2		3386.1 (-4.1)	3384.3 (-5.9)	6
		3408.2		3402.2 (-6.0)	3400.5 (-7.7)	5
pNZE3-DFALD	5643.4	3354.2	5647.8 (+4.4)	3351.1 (-3.1)	3350.9 (-3.3)	8
		3372.2		3368.0 (-4.2)	3371.0 (-1.2)	7
		3390.2		3390.8 (+0.6)	3394.2 (+4.0)	6
pNZE3-DFNAD	5644.3	3354.2	5652.9 (+8.6)		3357.3 (+3.1)	8
		3372.2		3370.1 (-2.1)	3374.3 (+2.1)	7
		3390.2		3387.3 (-2.9)		6
pNZE3-DFNLA	5642.4	3354.2	5656.0 (-4.4)	3357.7 (+3.5)	3357.5 (+3.3)	8
	5660.4	3372.2		3371.9 (-0.3)	3371.2 (+1.0)	7
		3390.2		3386.8 (-3.4)		6
pNZE-nis Δ(23-34)	4489.9	2139.0	4485.8 (-4.1)	2138.5 (-0.5)	n.d.	5
		2157.0		2154.6 (-2.4)		4
		2175.0		2170.7 (-4.3)		3

– no addition of NisP; + addition of NisP. n.d. not determined. The mass difference between the expected and the measured masses is displayed in brackets for each one of the possible dehydration statuses considered. In the case of misscleavage, the additional or lacking amino acids are displayed in brackets in the dehydration columns together with the number of dehydroamino acids.

Lanthionine ring-impaired nisin mutants

We designed variants of nisin containing only the first ring (nisin-CAAAA), lacking the first ring (nisin-C7A) and lacking all the rings (nisin-Cys-less). In order to discard an abnormal ring formation between Cys11 and an N-terminal dehydrated residue other than Thr8 in the mutant nisin-C7A, we designed the mutant nisin-ringA-less, where the first dehydratable residue is Thr8 and therefore we abolish the possibility of any aberrant ring formation. Additionally, we mutated the residues P4 to P1 in the leader peptide of nisin to create a VSLR cleavage sequence in nisin, nisin-CAAAA, nisin-C7A, nisin-ringA-less and nisin-Cys-less. We observed that wild-type nisin was

fully cleaved, independently of the cleavage sequence at the end of the leader peptide being ASPR or VSLR. In the other cases, although most of the cleavage took place in the right position, we were able to detect masses corresponding to aberrant cleavage by NisP (Table 2).

The mutant lacking all the cysteines (nisin-Cys-less) was produced in a very low amount, irrespectively of the cleavage site present. After the cleavage of nisin-Cys-less, diverse peaks with a mass corresponding to the cleaved and dehydrated peptide were detected with a small fraction of incorrectly processed nisin containing additional residues from the leader peptide (Table 2). Similarly, in the mutants nisin-CAAAA, nisin-C7A and nisin-ringA-less, small peaks corresponding to peptides cleaved at abnormal P1 or P2 positions in the leader peptide were also observed (Table 2). In the mutants nisin-C7A and nisin-ringA-less, a small fraction of nisin lacking the first amino acid(s) was also identified, suggesting cleavage at alternative positions.

Cleavage site mutants

Various amino acid residues were replaced at the N-terminus of the nisin core peptide (positions P1' and P2') to investigate the influence of these residues on the cleavage by NisP. We engineered positive and negative charges (nisin I1K, nisin T2K, and nisin I1D), a bulky amino acid (nisin I1W) and a hydrophobic amino acid in the second position of nisin core peptide replacing the dehydrobutyrine residue (nisin T2V) (Table 2). In all P1' and P2' mutants, we could detect inhibition of growth of sensor bacteria after activation with NisP. This indicates that these positions can tolerate a large variety of amino acids of very different nature and can still be cut by the specific protease and retain antimicrobial activity. It was possible to detect a small fraction of uncleaved peptide after the incubation of the mutant nisin I1D with the protease, which indicates reduced cleavage efficiency. The mutant nisin I1K rendered a small fraction of mature nisin with a mass difference consistent with the presence of Arg P1 in the mature peptide, indicating that the peptide is incorrectly cleaved behind ASP.

Additionally, we tested the efficiency of NisP to cleave other protease recognition sequences, namely Factor Xa, thrombin, endoprotease Glu-C, and enterokinase (DDDK and DDDDK sequences) (Table 2). NisP was able to cleave when a factor Xa or thrombin site was present. Surprisingly, the nisin mutant with a glutamic acid in the P1 position of the leader peptide (nisin V8) was cleaved, although only partially since uncut peptide was still visible after the experiment. The final amount of undigested peptide in case of the mutant nisin V8 was higher when the cleavage was performed at pH 6. The enterokinase sequences DDDK or DDDDK were not cleaved by NisP in the conditions tested. Noticeably, no unspecific cleavage in the core peptide of any of the tested mutants was observed in the conditions tested.

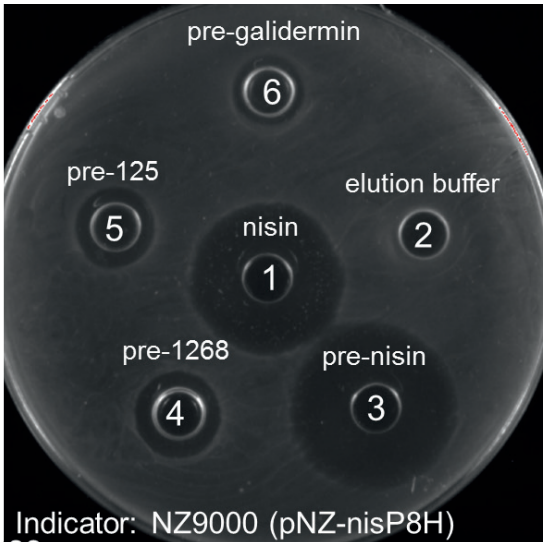

Figure 5. Antimicrobial activity of purified modified lantibiotics gallidermin, 1215 and 1268 against *L. lactis* NZ9000 (pNZnisP-8H). 1) commercial nisin; 2) elution buffer used during the purification, 3) purified prenisin; 4) pre-1268; 5) pre-1215; 6) pregallidermin.

Nisin mutants in the (D)FNLD box

Previously reported single alanine replacements of the characteristic DFNLD motif of type I lantibiotics leader peptide were studied.[14] In all cases, cleavage was taking place normally as shown by mass-spectrometry (Table 2) and antimicrobial assays (data not shown) were consistent with previous reports. [14]

Cleavage of different lantibiotic substrates

The substrate tolerance of soluble NisP was tested using a set of different lantibiotics. These were produced using the nisin modification machinery encoded in NZ9000 (pIL3BTC pNZE3-mutant) and purified by cationic exchange chromatography. For this purpose, NZ9000 (pNZ-nisP-8H) was used as a sensor strain and tested using gallidermin[38] and two lantibiotics detected in the genome of *Streptococcus pneumoniae*, PneA1 and PneA2 encoded in the genes *spr1765* and *spr1766*, respectively, which were heterologously expressed in *L. lactis* fused to the nisin leader peptide (Figure 5).[33] We could clearly determine that in the absence of NisP, none of the prelantibiotics tested, not even prenisin, was active against the sensor strain (data not shown). The activity was restored when a strain producing soluble NisP was employed. We could also notice that the NisP producing strain showed increased sensitivity to nisin (data not shown).

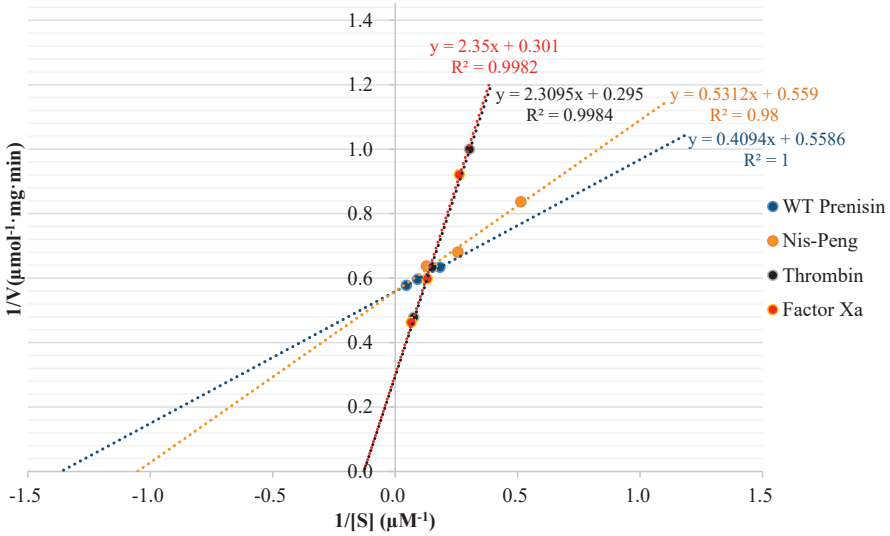

Figure 6. NisP kinetic parameters determination using Lineweaver–Burk plot. The substrate concentrations ranged between 1 and 25 μM.

Table 3. Kinetic characterization of the cleavage of several nisin leader peptide mutants.

Prenisin variants	Km (μM)	Vmax (μmol/mg/min)	Kcat (s^{-1})	kcat/Km ($M^{-1}s^{-1}$)
WT Prenisin (ASPR)	0.73±0.08	1.79±0.10	1.25±0.07	1.71×10^{6}
Nis-Peng (VSLR)	0.95±0.08	1.79±0.17	1.25±0.11	1.32×10^{6}
Throwbin (AVPR)	7.83±0.27	3.39±0.20	2.37±0.14	3.03×10^{5}
Factor Xa (IEGR)	7.81±0.01	3.32±0.10	2.33±0.07	2.98×10^{5}

Data indicate the mean value ± standard deviation.

Kinetic characterization of NisP

The substrate specificity and kinetic parameters of engineered soluble NisP were investigated by using WT prenisin (ASPR), nis-Peng (VSLR), nis-Thrombin (AVPR), and nis-Factor Xa (IEGR) as substrates. The *Km* and *Vmax* values were determined using Lineweaver-Burk plots (Figure 6). When wild-type prenisin was used as substrate, NisP exhibited the highest catalytic efficiency (*kcat/Km*=1.71×10^{6} $M^{-1}s^{-1}$) and affinity (*Km*=0.73 μM). In comparison with wild-type prenisin (ASPR), Nis-Peng (VSLR) showed a slightly increased *Km* value, an identical *Kcat* value and a comparable *kcat/Km* values (Table 3). For nis-Thrombin (AVPR) and nis-Factor Xa (IEGR), the 5–6 fold decrease in *kcat/Km* was ascribed to a 10–11 fold increase in *Km* and 2-fold increase in *Kcat* compared with that of wild-type prenisin (Table 3). In summary, soluble NisP displayed the highest catalytic efficiency to wild-type prenisin (ASPR), followed by

Nis-peng (VSLR). A 5-6 fold decreased catalytic efficiency was observed when Thrombin (AVPR) and Factor Xa (IEGR) cleavage sites were engineered.

Discussion

Active soluble NisP can be produced and purified

During the production of nisin, the protease NisP is exported and anchored to the cell wall in a sortase-mediated manner. In previous attempts to characterize the specificity of NisP, the *in vivo* cleavage was based on the detection of the antimicrobial activity and the detection of mature lantibiotic compared to that of the prelantibiotic.[54] The activity *in vitro* could be confirmed using membrane preparations of cells expressing NisP bound to the cell wall, which limited the assays. Therefore, we engineered a soluble NisP variant that could facilitate this task. Our initial hypothesis was that the removal of the sortase recognition sequence in NisP would abolish the binding, thus allowing for the purification of unbound protease in the supernatants (Figure 1). This approach failed to produce soluble NisP, maybe because NisP cannot fold properly or its solubility is reduced. Therefore, a second set of NisP mutants, trimmed at the C-terminus to remove the cell wall spanning helix, were designed. In this case, it was possible to produce detectable amounts of active NisP with the expected size in the supernatants. The activation of prenisin is unambiguously due to the action of this protease, since the strain with the empty vector was not able to activate prenisin. We also show that the purified protein could activate prenisin following SDS-PAGE electrophoresis, producing an inhibition halo around the band of the purified protease. These active NisP variants leave the subtilisin-like serine protease domain intact, and are more similar in size to other LanP-like proteases, which are naturally produced as soluble forms (EpiP[18] or PepP[19]) or have been heterologously produced as soluble proteases (ElxP[53]). Notably, *E. coli* was also able to produce mature NisP with the expected size in the supernatants (data not shown). This indicates that the secretion signal is correctly processed in *E. coli* and that the self-activation of NisP can happen. In previous reported attempts to express the full length NisP in *E. coli*, no detection of the protease by Coomassie staining was possible. However, isotope-labeled methionine showed the production of a processed NisP with a size consistent with the removal of the signal sequence[16], as was observed in our study. This cell wall attached NisP could activate prenisin when cell extracts of induced *E. coli* were used.[16] Importantly, the activity of NisP was not abolished by the addition of a C-terminal poly-histidine tag or a poly-lysine tag.

NisP tolerates mutations at the N-terminus of mature nisin

A homology modeling study on NisP based on the comparison with other proteases from the same family predicted an active site with a binding pocket that could fit

6 amino acids (P4, P3, P2, P1, P1' and P2').[43] It predicts a strong interaction between the arginine in the position P1 with the protease mediated by electrostatic interactions with two aspartates in the enzyme that limits the amino acids in the positions P1' and P2' to be small hydrophobic amino acids.[43]

The presence of amino acids of diverse nature in the positions P1' and P2' of nisin shows that these residues can be mutated without altering the cleavage by NisP. It was anticipated that the binding of dehydrobutyrine in position P2' was less important than the interaction of Ile in position P1' with a hydrophobic groove in NisP.[43] Some mutants like I1W were previously shown to be cut by NisP using only the antimicrobial activity as evidence.[55] Here we show that it is fully processed under the experimental conditions used. We could observe that an additional positive charge in the mutant I1K produced a small additional peak detected by mass-spectrometry consistent with a cleavage between positions P2 and P1, suggesting that the accumulation of positive charges favors a non-specific cleavage by NisP. Bulky or charged amino acids are well tolerated and the dehydrobutyrine in the second position, or a previously engineered dehydroalanine[55], are not the requirement for cleavage.[43]

Lanthionine rings in nisin are not essential for cleavage by NisP

Additional studies have suggested the requirement of lanthionine rings as a prerequisite for the substrate recognition.[27] We engineered various mutants with altered lanthionine rings. NisP is supposed to cut in the VSLR+QP sequence in its N-terminus[16] and VSVR+S at the C-terminus[21], which lack lanthionine rings. Therefore, we hypothesize that a higher tolerance could be expected for mutants containing the engineered VSLR site in the leader peptide. Thus, we engineered in all the mutants with modified lanthionine ring topology the self-activation site proposed for NisP, the VSLR cleavage sequence. In previous work, a nisin mutant (A-4V,P-2L,I1Q,T2P, positions P4, P2, P1' and P2' according to Schechter-Berger nomenclature), containing the whole self-cleavage site of NisP was previously reported.[14,16] We show here that VSLR+IT is also cleaved by NisP. The cleavage pattern observed in the mutants with different number of rings shows that the presence of lanthionine is not essential for the cleavage, since nisin Cys-less seems to be cleaved and so is nisin CAAAA. A similar situation was observed with the cleavage *in vitro* of unmodified pre-epilancin 15X and unmodified pre-epidermin with the specific proteases ElxP and EpiP, respectively.[18,53] This is in disagreement with previous work using whole cells expressing cell-attached NisP that suggests the requirement of lanthionine rings for the cleavage reaction.[27] This discordance can be due to the amount of NisP expressed at the cell surface which could be too small compared to the addition of purified NisP, the additional replacements in nisin (S-6P, P-2L, positions P6 and P2, respectively) and/or the shorter incubation time used. It has been already shown that the binding affinity can be much higher for

the fully modified prelantibiotic than for the unmodified one.[18,44] Our results also indicate that although the lanthionine rings are not essential for the cleavage, they might favor the correct interaction with the protease and positioning of the substrate in the active site since small amounts of prenisin with either additional residues of the leader peptide or lacking some amino acids of the core peptide were visible, indicating occasional misprocessing. NisP production involves self-activation after the N-terminal signal peptide and cleavage at the C-terminus for optimal nisin production. Thus the promiscuity demonstrated in this study is also related to NisP expression itself (which recognizes different (auto) processing sites). This can be exploited for specific peptide cleavage at various slightly different sites, when producing variant lantibiotics behind the nisin leader peptide (Supplementary Figure 1).

NisP cleaves in all tested protease sites, except in the two enterokinase sites

As mentioned before, the active site of NisP interacts with the last 4 residues of the leader peptide.[43] We modified these last positions in the leader peptide of nisin generating a set of diverse protease substrates. In such a way, we could investigate the specificity of NisP and simultaneously compare NisP with other proteases for the cleavage of the leader peptide in molecules modified using nisin biosynthesis machinery (*vide infra*).

The diverse protease sites engineered in the leader peptide prove that, except for the two enterokinase sites, all the other cleavage sequences tested could be cleaved by NisP, albeit with different efficiencies. Our kcat/Km obtained for wild-type nisin is in line with previous results in literature, even though a different buffer is used.[44] The largest differences are noticed in the kinetic parameters of nis-Thrombin and nis-Factor Xa, with a 5–6 fold decrease in the catalytic efficiency (Table 3). Although the cleavage efficiency in these cases is reduced, this can be counteracted using prolonged incubation times or a higher enzyme dose, to yield full cleavage (data not shown). It is not surprising that the very similar thrombin site can be cleaved (AVPR *vs* ASPR in the wild-type) although the reduction in the catalytic efficiency points at the favored cleavage with a hydrophilic residue in the P3 position that can interact with the solvent out of the protease active site.[43] The replacement of ASPR with the factor Xa cleavage site IEGR introduces additional changes and a negatively charged residue, but is still rather efficiently cleaved by NisP. The presence of negative residues in position P3 is common in other type I lantibiotics processed by a LanP enzyme such as gallidermin or epidermin. In contrast, the mutant A-4D[54] is not fully cleaved. The predicted interaction partner in the enzyme is a small hydrophobic pocket and therefore the mutation A-4D could not fit well into this cleft.[43] Additionally, a weak hydrogen bond interaction between Ser P3 and NisP was modeled, whereas in EpiP the presence of lysine residues around the catalytic site could favor a stronger interaction with the negative charge in epidermin leader peptide.[43] Our results support the idea that a small hydrophobic amino acid in

the P4 position and a polar or charged amino acid in the P3 position may be important for the correct positioning of the leader peptide into the active site of NisP.

The highly conserved proline in the P2 position in the leader peptide of type I lantibiotics could play an important role in determining the local structure of the leader peptide and facilitating the access of the protease active site to cleave the mature prelantibiotic. It is also related to production levels and transport.[56] Our data and the self-cleavage sequences in NisP and several other Pro mutants in the position P2[14,21,27,54] clearly shows that proline is not essential for the cleavage (Figure 1B).

The highly conserved arginine in the P1 position establishes a strong ionic interaction with aspartate residues in the model structure of NisP.[43] This position shows certain flexibility, although the replacements R-1Q, which is present in other type I lantibiotics such as subtilin, Pep5 or epilancin 15X, and the mutation R-1E present in the mutant nisin V8, dramatically reduces the cleavage efficiency.[54]

Single alanine mutations in the (D)FNLD box do not hamper cleavage by NisP

The (D)FNLD box in lantibiotic leader peptides has been shown to be essential for the optimal interaction with the modification enzymes in nisin biosynthesis machinery and other type I lantibiotics.[2,6,8,9,12,14,15] Previous works reported the impact of various mutations in this region on the modification extent of nisin monitored by mass-spectrometry and activity tests. However, the activity was measured after activation with trypsin.[9,12,14] We considered this DFNLD motif as a possible recognition sequence also for the protease NisP. This was previously considered in the homology modeling study in view on unaligned fragments of NisP that were considered important for the binding to the substrate prenisin.[43] All the single alanine replacements we tested as substrate for NisP could still be cleaved correctly. This suggests that either this is not a recognition site for NisP or that single alanine mutations are not enough to disrupt the interaction. Similarly, replacements in the region between the FNLD box and the ASPR cleavage sequence of the leader had no great impact on the production of nisin, as was published before.[54]

NisP as a tool to release diverse lanthionine-containing peptides

The plug-and-play production system for lantibiotics developed in the last years constitutes a robust platform for the production of diverse lanthionine-containing peptides.[31-33,38] Moreover, prelantibiotics have no biological activity and higher yields can be achieved (Valsesia et al., 2007).[35] Thus, a leader peptidase with broad specificity for the peptide moiety behind the cleavage site is desired. A cheap peptidase capable of working directly in culture broth is preferred since this can facilitate efforts during high-throughput screening approaches. NisP fulfills these two criteria and

constitutes a valuable tool for the cleavage of the leader peptide in the production of lanthionine-stabilized peptide hormones[26] as well as fully modified non-cognate prelantibiotics (Figure 5). The increased sensitivity compared to the plasmid free strain can be due to stress caused by the production of the protease (data not shown). The attempts to express the lantibiotic protease ElxP in the wild-type producer also caused toxicity issues.[57]

Various proteases are commonly used for the cleavage of recombinant proteins with an affinity tag attached, such as TEV protease, enterokinase, Factor Xa, or thrombin. Some of them cleave at the end of their recognition sequence, whereas some others cleave in between, therefore adding extra residues after the cleavage site (i.e. TEV protease). The latter proteases are disadvantageous for our purposes since they leave some amino acids behind that cannot be removed. Factor Xa can tolerate most amino acids behind the cleavage sequence I(D/E)GR with the exception of lysine and proline. However it needs very controlled conditions and commonly cleaves incompletely making its biotechnological application costly. Thrombin, although it cleaves in the middle of the recognition sequence, shows some flexibility in the residues behind the cleavage site and tolerates different hydrophobic amino acids.

We tested different proteases and showed that NisP, the endoprotease Glu-C, thrombin and trypsin are able to cleave in culture conditions. Factor Xa could not activate prenisin in these conditions. Trypsin and the endoprotease Glu-C are very unspecific, since they can cleave behind positive or negative residues within the propeptide, respectively. This is a limitation to their applicability in many cases. However, the endoprotease Glu-C could be suitable in specific cases due to the low abundance of negative residues in lantibiotics and the pH conditions during cleavage can favor the cleavage selectivity for either aspartate or glutamic acid.

Conclusions

Taken together, the results presented here suggest that the protease NisP has greater substrate tolerance than previously anticipated. The exact recognition motif in the leader peptide of nisin for the binding of NisP is not yet completely resolved, although our results agree with the predicted ionic interaction between Arg in position P1 and NisP as a requirement for efficient cleavage and confirm that the presence of lanthionine rings is not mandatory for the cleavage. The determination of the structure of NisP and the kinetics of the cleavage reaction of the different mutants could surely shed light on the residues more directly involved in the binding to NisP. These insights should help to expand the biotechnological potential of NisP as a general tool for the cleavage of proteins with and without lanthionine residues. Our results also show that among all the proteases tested, NisP is the most suitable and inexpensive candidate for the

activation of diverse lantibiotics or thioether-stabilized peptides, produced with the nisin leader peptide and the modification machinery of nisin.

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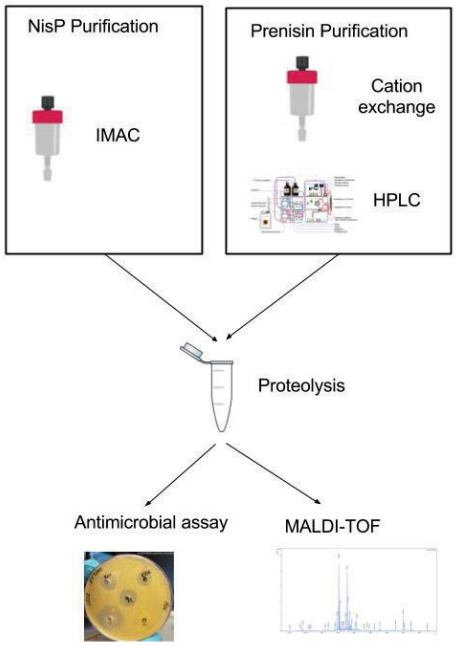
Supplementary Table and Figure



Supplementary Figure 1. Schematic view of a possible workflow for the biotechnological application of NisP using nisin as an example.

Supplementary Table 1: Primers used in this study

Name	Sequence	Purpose
nisPbsphfwd	CGT GAG TCA TGA AAA AAA TAC TAG GTT TCC	Amplification of NisP
nisP8KXbarev	TCG TGT CTA GAT TAT TTC TTT TTC TTT TTC TTT TTC TTT TGA CTT CGT ACA GAA ACA GC	Amplification of NisP without sortase sequence and 8-Lys
nisP8HXbarev	TCG TGT CTA GAT TAA TGG TGA TGG TGA TGG TGA TGG TGT TGA CTT CGT ACA GAA ACA GC	Amplification of NisP without sortase sequence and 8-His
nisP8KSacIr	TCGT GGA GCT CT TAC TTT TTC TTT TTC TTT TTC TTT TTA TCT GTA TCT AAG CTA AAA GC	Amplification of truncated NisP without cell wall helix and with 8-Lys
solnisP8HSacIr	TCGT GGA GCT CT TAA TGG TGA TGG TGA TGG TGA TGG TGA TCT GTA TCT AAG CTA AAA GC	Amplification of truncated NisP without cell wall helix and with 8-His
solnisPcontrol	TCGT GGA GCT CT TAA TCT GTA TCT AAG CTA AAA GC	Amplification of truncated NisP without cell wall helix
nisPrev2	GAC AAT ATC ACT TGG ATT TCC	sequencing
nisPrev2	GTT GTG CCA GCA GGA GC	sequencing
nisP8K/H	GA CTT CGT ACA GAA ACA GC	sequencing
pNZE3Emf	CAA TTC CTT AAA ACA TGC AGG	sequencing
pNZE3revMML	CAA TCA AAG CAA CAC GTG C	sequencing
C-lessH6-less	TC TAG AAG CTT ATT TGC TTA CGT GAA TAC TAG CAT GAG C	Removal of the his-tag in nisin C-less
NisPC7A-rev	GCT AGC GAA ATA CTT GTA ATA CGA AGT GAA ACA CCT GAA TC	Construction of nisin C7A-VSLR
NisPC7A-fwd	GTA TTA CAA GTA TTT CGC TAG CTA CAC CCG GTT GTA AAA CAG G	Construction of nisin C7A
nisVSLRfwd	CTACGCATTACAAGTATTTCGC	Creation of the VSLR cleavage sequence
nisVSLRrev	ACT TAC ACC TGA ATC TTT CTT CG	Creation of the VSLR cleavage sequence
P-for	AGTATTTCGCTATGTACACCCGGTTG	Mutation of residues I1 or T2 of nisin
P-IK-Rev	TTTAATACGAAGTGAAACACCTGAATCTTTCTTCGAAAC	Mutation T2K
P-KT-Rev	AGTTTTACGAAGTGAAACACCTGAATC	Mutation I1K
P-WT-Rev	AGTCCAACGAAGTGAAACACCTGAATC	Mutation I1W
P-DT-Rev	AGTATCACGAAGTGAAACACCTGAATC	Mutation I1D
P-IV-Rev	CCTGACTTTTTCTTACGAAGTGAAACACCTGAATC	Mutation T2V
NisPC7A-ASPR-rev	TAGCGAAATACTTGTAATACGTGGTGATGCACCTGAATC	Construction of nisin C7A-ASPR
ringAdel-fwd	TGTTGCAATTGCGCTAGCTACACCCGGTTGTAAAACAGG	Mutation of dehydratable residues N-terminal to T8
RingAless-ASPR-rev	TAG CGC AAT TGC AAC AAT GCG TGG TGA TGC ACC TGA ATC	Mutation of dehydratable residues N-terminal to T8
RingAless-VSLR-rev	TAG CGC AAT TGC AAC AAT ACG TAA TGA AAC ACC TGA ATC	Mutation of dehydratable residues N-terminal to T8

CHAPTER 3

Analysis of modular bioengineered antimicrobial lanthipeptides at nanoliter scale

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I contributed to the purification and determination of the MICs of the peptides and to parts of the writing.

Abstract

The rise of antibiotic resistances demands the acceleration of molecular diversification strategies to inspire new chemical entities for antibiotic medicines. We report here on the large-scale engineering of ribosomally synthesized and post-translationally modified antimicrobial peptides carrying the ring-forming amino acid lanthionine. New-to-nature variants featuring distinct properties were obtained by combinatorial shuffling of peptide modules derived from 12 natural antimicrobial lanthipeptides and processing by a promiscuous post-translational modification machinery. For experimental characterization, we developed the nanoFleming, a miniaturized and parallelized high-throughput inhibition assay. Based on a hit set of >100 molecules, we identified variants with improved activity against pathogenic bacteria and shifted activity profiles, and extrapolated design guidelines which will simplify the identification of peptide-based anti-infectives in the future.

Introduction

Close to 75% of all approved antibiotics have their origin in nature, highlighting the importance of natural products for drug development[1]. However, identifying new lead molecules from this pool, preferably with novel modes of action, is becoming increasingly difficult[2–5]. The further development of existing molecules by chemical diversification, another well-established strategy[6], delivers only limited structural novelty. More recently, biological diversification of enzymatically produced natural products (such as non-ribosomal peptides or polyketides) has been introduced. This strategy relies on the recombination of the involved enzyme clusters and delivered promising leads[7,8]. However, the lack of insight into how to generate sufficient modularity for efficient enzyme shuffling and the restricted experimental throughput to explore large combinatorial spaces limit the impact on drug development[9,10].

In practical terms, molecule diversification in bioengineering approaches becomes much easier if the antimicrobial molecule is a gene product itself rather than the catalytic result of several gene products whose engineering has to be carefully coordinated. One such example of a gene-encoded natural product are ribosomally produced and post-translationally modified peptides (RiPPs)[11]. Here, application of the well-developed methods of DNA synthesis and modification allow direct synthesis of highly diverse peptides, which are then further modified with the functionally important post-translational modification machinery. Among RiPPs, the class of antimicrobial lanthipeptides (i.e. lantibiotics) represents a rich source for promising leads against Gram-positive bacteria. The best-known representative, nisin, already has as a long history as a food preservation agent and others entered recently into clinical development for infectious diseases[12,13]. Lantibiotics carry ring-forming amino acids (lanthionine and methyllanthionine) that result in small peptide stretches that are considerably restricted in their rotational degree of freedom (Figure 1a) and are introduced by an, often promiscuous, post-translational modification (PTM) machinery (Supplementary Figure 1), suggesting the possibility for diversifying the peptide backbone, while still enabling modifications[14–19].

Lantibiotics commonly bind to the bacterial cell wall precursor lipid II, inhibiting cell wall formation and often also induce pore formation in the cytoplasmic membrane of their target cells[20]. They feature a similar blueprint encompassing the location of functional elements (lipid II binding and membrane piercing) as well as the organization of the thioether rings within the peptide backbone (Figure 1a). However, the ring structures themselves vary considerably in size and primary structure over different peptides and the peptides display highly different degrees of activity towards target strains[20]. This diversity raises the intriguing opportunity of large-scale molecular shuffling to obtain novel functionality based on modules broadly organized along

ring structures and other functional segments (Figure 1b). In order to overcome the otherwise prohibitive issue of sorting through large numbers of peptide variants, we miniaturized Fleming's inhibition zone assay[21] by evaluating the result of coculturing RiPP producers and a sensor strain at nanoliter scale ("nanoFleming") in nanoliter reactors (nLRs) and at high-throughput[22–25]. Here we present the results of shuffling 33 lantibiotic peptide modules with natural or synthetic background yielding a library of 6,000 putatively active structures. Screening of the library with the nanoFleming platform followed by detailed characterization resulted in a set of 11 antimicrobial lanthipeptides that showed improved antimicrobial activity over wild-type peptides or were able to bypass resistance mechanisms.

Results

Design of a combinatorial lanthipeptide library

Peptide modules were recruited from twelve natural lantibiotics (Supplementary Table 1) representing broadly linear peptides with lipid II binding and pore formation (type A lantibiotics, including nisin), globular peptides with large and intertwined rings with lipid II affinity, but without perforation capacity (type B lantibiotics, e.g. actagardine), and peptides with a lipid II binding and a pore-forming subunit (two-component lantibiotics, e.g. haloduracin). The peptides were modularized according to rotationally restricted regions comprised of one single or two interwoven thioether rings and flexible, interconnecting ("hinge") regions. For nisin, we identified five modules (binding modules B1 and B2 involved in lipid II binding and pore modules P1 to P3 involved in pore formation, Figure 1a), extracted a further 23 modules from the remaining 11 lantibiotics, and allocated those to positions B1 to P3 (Figure 1b). The set was completed with non-natural interconnecting hinge modules (P2) of different lengths and charges to increase the likelihood for activities against different target strains[26] and a placeholder at P1 to represent natural lantibiotics missing this module (e.g. gallidermin). Finally, we limited B1 to modules from nisin and gallidermin as this module is critical for PTM by the nisin biosynthetic machinery (*vide infra*)[17]. Next, we generated new-to-nature peptides by randomly combining one module of each of the five groups, employing chemical synthesis of the peptide-encoding DNA and a split-and-mix approach to implement modular recombination (Supplementary Figure 2). PTM and export of lantibiotics are dependent on an N-terminal leader peptide; therefore the resulting DNA library of 6,000 combinatorial variants was fused to the leader peptide of nisin and overexpressed in a *Lactococcus lactis* also expressing the nisin PTM machinery NisBTC (i.e. including the nisin export function NisT but excluding the protease NisP required for peptide activation by cleavage of the leader peptide)[19] (Figure 1c).

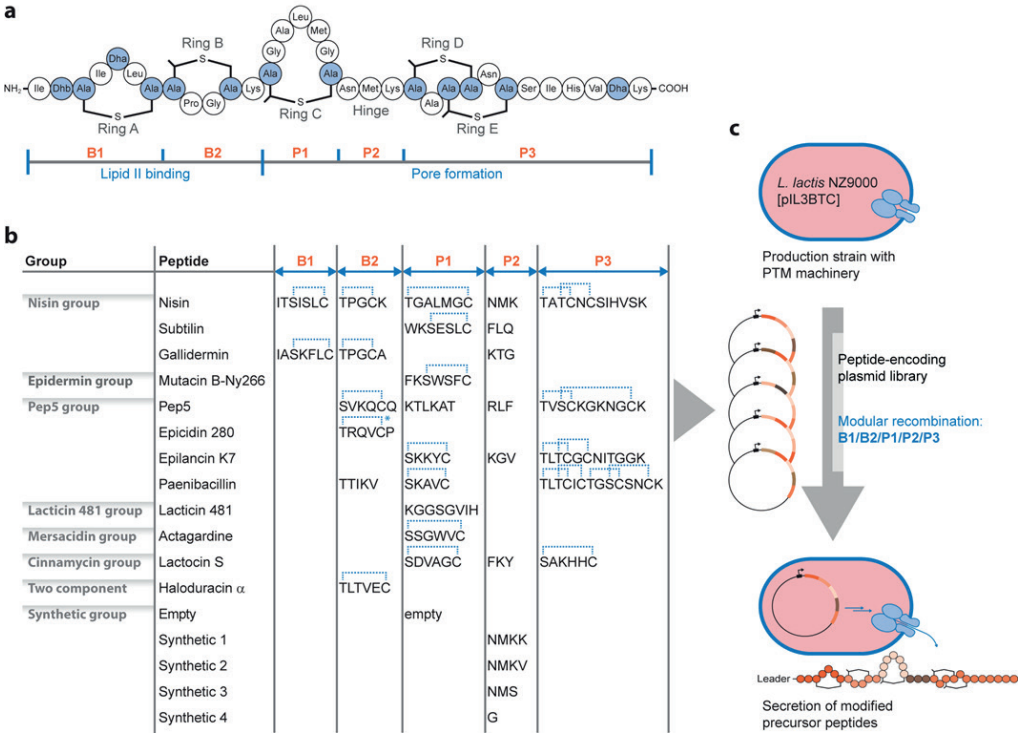

Figure 1. Modular assembly of antimicrobial lanthipeptides. (**a**) Module-reflecting blueprint of the lantibiotic nisin with the thioether rings A to E and the hinge region translated into five peptide modules B1 to P3. (**b**) Segmentation and assignment of 12 natural lantibiotics into modules. Known (*=estimated) thioether rings are indicated. A fictitious P1 module (marked as "empty") is used in our design to mimic shorter lantibiotics (e.g. epidermin and gallidermin) that lack a sequence connecting B2 to the P2 region. (**c**) The different modules were shuffled by DNA synthesis retaining the sequence B1/B2/P1/P2/P3 and the library of 6,000 variants was overexpressed in the PTM-competent secretion host *L. lactis* NZ9000 [pIL3BTC].

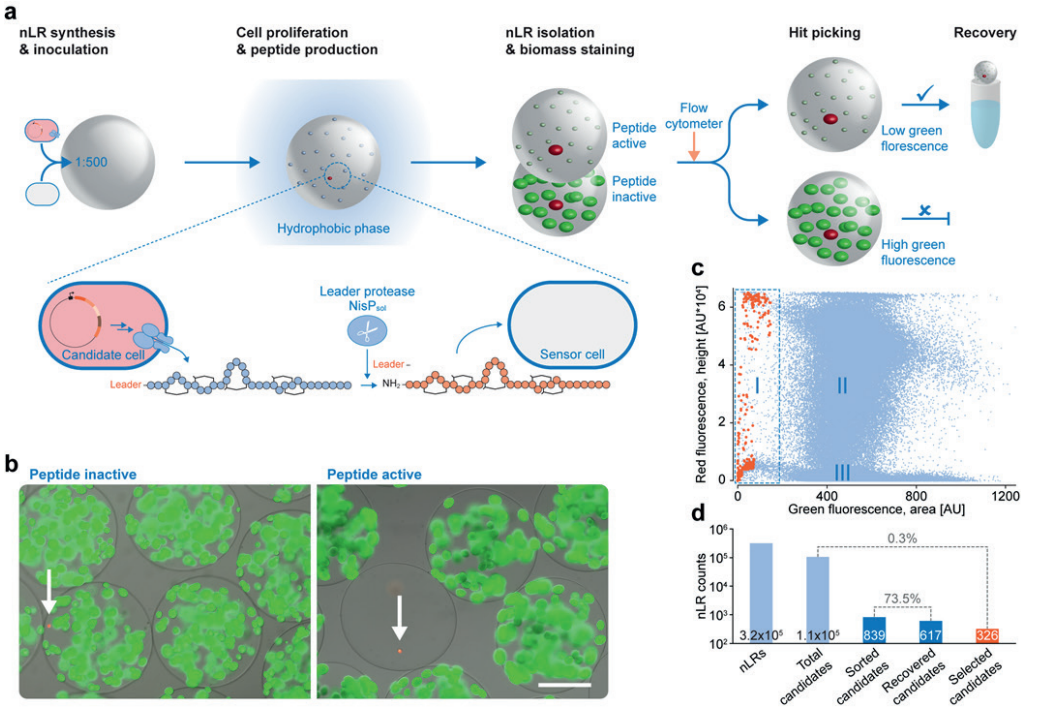

Figure 2. Discovery platform for antimicrobial peptides. (**a**) nanoFleming workflow: Library (peptide secreting and a red fluorescent protein mCherry producing *L. lactis*, in red) and sensor (*M. flavus*) cells are encapsulated in nLRs at 0.3 and 150 cells nLR^{-1}, respectively. Next, nLRs are soaked with growth medium containing the leader-specific protease NisPsol, resuspended in a hydrophobic phase, and incubated. Depending on the specific activity of the secreted peptides, the colocalized sensor cells may either continue to grow or experience different degrees of growth inhibition. After incubation, nLRs are recovered from the hydrophobic phase, stained and sorted based on the amount of sensor strain per nLR. Candidate cells in nLRs with little sensor biomass are recovered. (**b**) Overlay of bright-field and epifluorescence microscopic images of nLRs after incubation. Left: candidate colony (red, arrow) not secreting an active lantibiotic results in large sensor colonies (green). Right: candidate colony secreting an active lantibiotic (here: nisin). Scale bars: 200 µm. (**c**) Dot plot of the results from flow cytometric analysis of nLRs. 3.2×10^5 nLRs (blue dots) inoculated with 1.1×10^5 candidate cells (18-fold oversampling of the library). Red fluorescence indicated the size of the microcolony of candidate cells (region I+II = large; region III = small/absent) and green fluorescence indicated biomass of sensor cells (region I = low; region II+III = high). (**d**) Summary of isolated peptide candidates over the nanoFleming workflow.

Development of an inhibition assay at nanoliter scale

To enable rapid bioactivity assessment of the library peptides, we developed the nanoFleming high-throughput platform for antibiotic screening. We used small alginate hydrogel compartments (500 µm diameter, volume 65 nL, hence nLRs) for bacterial growth, peptide production and bioactivity testing. In a typical experiment, on average 0.3 library cells were encapsulated per nLR together with 150 cells of the sensor strain *Micrococcus flavus*. The nLRs were soaked in growth medium containing the soluble form of the protease NisP (NisPsol)[27] required for the activation of secreted peptides (Supplementary Figure 3). Incubation, conducted in a hydrophobic phase to prevent cross-talk between nLRs, allowed for the growth of library and sensor cells and peptide production. After incubation and recovery from the hydrophobic phase, the nLR-embedded biomass was stained with the fluorescent dye SYTO 9 and nLRs with no or only very little biomass, indicating effective prevention of sensor strain growth, were isolated (Figure 2a and Supplementary Figure 4). To characterize the assay, we first compared the inhibition of nLR-embedded sensors in the presence and absence of colocalized prenisin-secreting cells and found that candidate strains secreting prenisin, but not a non-secreting control strain, efficiently inhibited the growth of the sensors (Figure 2b and Supplementary Figure 5). We also observed a higher sensitivity of the nanoFleming assay when compared to standard inhibition zone assays (Supplementary Figure 6). This corroborated the suitability of the miniaturized assay for the identification of compounds in screening campaigns where production levels of the active substance might frequently be low.

Library screening and hit verification

Next, we screened the combinatorial peptide library using the nanoFleming platform (Figure 2c). Out of 3.2×10^5 nLRs, we isolated 839 nLRs (0.8%) containing very low levels of sensor biomass. The nLRs were spotted on agar plates and 617 of the embedded candidate strains (73.5%) could be regrown. We selected the 326 candidates that had shown the lowest green fluorescence in the screen for further processing.

The peptide-encoding DNA sequences of all isolated clones were determined and 205 unique peptide variants were characterized with respect to production level and antimicrobial activity. Each clone was grown in liquid culture, the secreted peptide was precipitated and the leader was fully cleaved with NisPsol. As only the sequence of the putatively antimicrobial core peptides but not the leader sequence varied, the production level and the fraction of correctly cleaved peptide could be estimated on the basis of the leader concentration by HPLCMSMS. Next, the activity of the mixture was analyzed with a conventional inhibition zone assay[28] against *M. flavus* (Supplementary Figure 7) and a panel of model pathogens (Supplementary Figure 8). The activity data as well as the production levels were compared to nisin as reference. From the group of

205 isolated clones, we identified 126 peptides that showed reproducible halo formation against *M. flavus*. Based on the DNA sequence data, heavily (modules from up to five different parents combined in a single peptide) as well as mildly shuffled antimicrobial peptides had been generated.

Design guidelines for bioactive lanthipeptides

We next set out to identify guidelines for the design of bioactive molecules based on the activity and secretion level of the 205 unique peptides obtained in the initial screen (126 positive, 79 negative). In order to ensure NisP cleavage, only two modules had been included for permutation at B1. Both were found in the screening hits and the corresponding peptides displayed considerable activity and production levels (Figure 3). At B2, the modules derived from gallidermin and nisin were clearly overrepresented in the fraction of the isolated peptides and seemed to facilitate processing and secretion as compared to the remaining 4 options. Furthermore, all peptides bearing these two modules had a rather high activity, possibly indicating efficient processing of the peptide by the NisBTC PTM machinery[17]. At P1, the structural variety found within the subset of efficiently produced peptides was much larger than at B2 and modules derived from actagardine, nisin, paenibacillin, pep5 and subtilin were found. Similarly, modules from actagardine, nisin and paenibacillin were found in active bacterial variants. For P2 (hinge region), all modules showed production (at variable mean levels) and were represented among bioactive peptides. Similar results were observed for P3: All 5 possible modules were among the population of analyzed peptides, but we again observed a clear overrepresentation of the nisin-derived module. Still, all modules tested for P3 were included in bioactive peptides. Taken together, 22 of the 33 modules that had been shuffled were afterwards identified in newly generated bioactive peptides. These results indicate that antimicrobial lanthipeptides can be assembled by combinatorial recombination of peptide modules and that despite a considerable variation of the amino acid sequence, most of these modules can become part of novel and bioactive peptides.

Minimal inhibitory concentrations against pathogens

Based on their activity in the preliminary assays and their modular diversity, we selected 61 peptides for further characterization. To facilitate the purification of the peptides in large quantities, we integrated a His_6-tag into the leader peptide. Modified precursor peptides were then purified via immobilized metal ion affinity chromatography (IMAC), the leader peptide was removed and the core peptides were further purified by RP-HPLC. For 31 peptides, this pipeline allowed purification of sufficient material to determine the minimal inhibitory concentration (MIC) against *M. flavus* and a panel of seven Gram-positive pathogenic strains, including *Streptococcus pneumoniae*, two

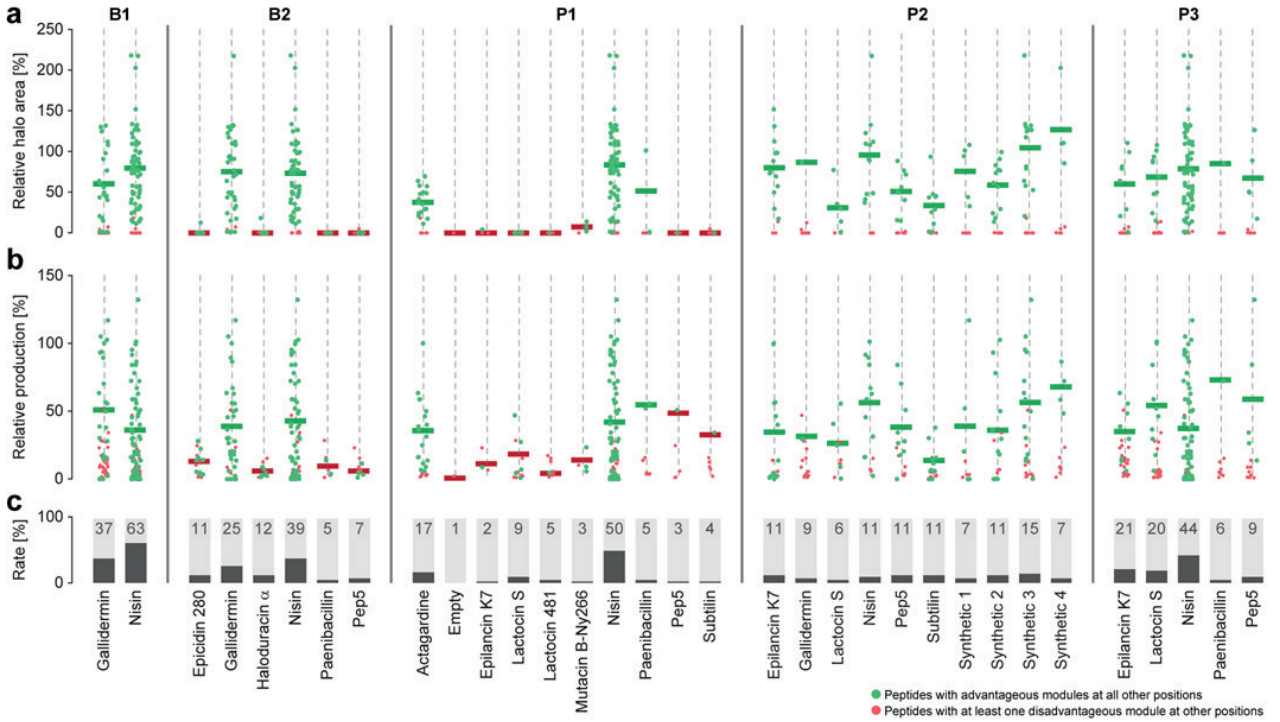

Figure 3. Characterization of screening hits. (**a**) Relative antimicrobial activity as a function of module nature and position. Each dot represents a peptide that was tested and had the specific module present in that position. Vertical bars: Mean of activities of all tested peptides with this module. Bar color indicates classification as advantageous (green, leading to a bioactive molecule) or disadvantageous (red, not leading to a bioactive molecule) module. Mean values were calculated from only those peptides which had advantageous modules at all other positions (green dots). (**b**) As in (a), but for relative production level. (**c**) Fraction of the module in all isolated variants (n = 205). All peptides were precipitated in duplicate (n = 2) and antimicrobial activity and production levels were quantified in triplicate. Each dot represents the mean of those measurements.

Table 1. MICs (μg mL^{1}) of combinatorial peptides against pathogens.

MIC [μg mL^{1}]	Module combination					*S. pneumoniae* TIGR-4	*S. pneumoniae* D39	*S. aureus* CAL (MRSA)	*S. aureus* MW2 (MRSA)	*E. faecalis* LMG 16216 (VRE)	*E. faecium* LMG 16003 (VRE)	*B. cereus* ATCC 14579
Peptide	B1	B2	P1	P2	P3							
1 SC5.2712	Gallidermin	Gallidermin	Nisin	Nisin	Nisin	0.53	0.42	7.36	38.90	7.36	7.36	37.85
2 SC5.1421	Gallidermin	Gallidermin	Nisin	Syn.1	Nisin	2.01	2.29	3.73	2.80	1.40	11.20	44.80
3 SC5.2930	Gallidermin	Nisin	Nisin	Syn.3	Nisin	0.84	1.13	26.97	17.98	290.0	6.74	26.97
Gallidermin						0.90	1.81	11.30	13.56	27.12	54.25	37.29
Nisin						4.21	4.21	9.86	10.91	6.68	7.72	12.19

MICs of a selected set of combinatorial peptides against a panel of seven Gram-positive model pathogens. Values that are improved in comparison to one of the wild-type peptides nisin or gallidermin are highlighted in grey. Values are means of three MIC experiments (n = 3).

methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus faecalis*, vancomycin resistant *E. faecium* and *Bacillus cereus*.

We observed a large MIC-range of the purified peptides against the screening strain *M. flavus*, with many of the peptides exhibiting an MIC of <0.5 μg mL^{-1} (Supplementary Table 2 and Supplementary Figure 9). Furthermore, we identified peptides with improved activity against one or more of the pathogenic reference strains when compared to nisin and gallidermin (Table 1). Nisin seems particularly active against both Enterococci and both Staphylococci strains and gallidermin against the two Streptococci strains. When combining the lipid II binding moiety B1 and B2 of gallidermin with the pore-forming modules P1-P3 of nisin, the combinatorial peptide 1 showed improved activities against Streptococci when compared to nisin and improved activities against one of the two Staphylococci and the two Enterococci when compared to gallidermin. The similar variant peptide 2 with an additional module exchange in P2 showed even better activity against the two Staphylococci and one Enterococci. Certain module combinations also led to peptides with strongly reduced activities against a specific strain, but retained a high specific activity against others, suggesting a possible increase in selectivity. For example, peptide 3 displayed good bioactivity against most strains of the test panel but activity against one of the Enterococci was strongly reduced when compared to nisin or gallidermin (Table 1).

Bypassing lantibiotic resistance mechanisms

Two specific microbial defense mechanisms against nisin are characterized[29]. One is constituted by the nisin immunity machinery, which is present in nisin-producing

Table 2. MICs (μg mL^{1}) of combinatorial peptides against nisin resistant strains.

a									
MIC [μg mL^{1}]		Module combination					*L. lactis*	*L. lactis*	
Peptide		B1	B2	P1	P2	P3	NZ9000	NZ9803	Immunity
1	SC5.2712	Gallidermin	Gallidermin	Nisin	Nisin	Nisin	0.05	1.30	25
3	SC5.2930	Gallidermin	Nisin	Nisin	Syn.3	Nisin	0.12	1.90	15
4	SC5.1659	Nisin	Nisin	Nisin	Syn.2	Nisin	0.80	12.00	14
5	SC5.1536	Nisin	Nisin	Nisin	Syn.4	Nisin	0.13	3.60	27
6	SC5.0925	Nisin	Gallidermin	Nisin	Syn.3	Nisin	0.03	4.00	132
Nisin							0.03	30.90	1029

b									
MIC [μg mL^{1}]		Module combination					*L. lactis* NZ9000	*L. lactis* NZ9000	
Peptide		B1	B2	P1	P2	P3	[pEmpty]	[pNSR]	Resistance
7	SC5.0718	Nisin	Nisin	Nisin	Pep 5	Lactocin S	0.65	0.65	0
8	SC5.2096	Nisin	Nisin	Nisin	Nisin	Pep 5	0.73	0.73	0
9	SC5.0364	Nisin	Nisin	Nisin	Syn.3	Pep 5	2.15	2.15	0
10	SC5.2354	Nisin	Nisin	Nisin	Syn.4	Paenibacillin	6.90	6.90	0
11	SC5.0479	Nisin	Nisin	Nisin	Syn.2	Epilancin K7	0.86	1.73	1
Nisin							0.08	1.63	19

*(**a**) MICs of a selected set of nisin-alike combinatorial peptides against a strain carrying the nisin immunity cluster on the genome (L. lactis NZ9803) and comparison to a strain that does not carry the cluster (L. lactis NZ9000). (**b**) MICs of a selected set of combinatorial peptides with various C-termini against a strain over-expressing the nisin resistance protein from a plasmid (NZ9000 [pNSR]) and comparison to a strain that carries an empty plasmid (NZ9000 [pEmpty]). Immunity and resistance are given as relative change (x/y-1). Values are means of three MIC experiments (n = 3).*

strains. This is composed of the proteins NisI and NisFEG, which prevent nisin binding to lipid II. The second mechanism is the nisin resistance protein (NSR) present in non-producing (pathogenic) strains. NSR works as a peptidase that cleaves the linear C-terminus of nisin (last 6 amino acids) after the interwoven rings DE and increases the MIC 20-fold. In the latter case, ring E is crucial for recognition[29,30]. We have identified both, peptides for which the natural nisin immunity system showed only reduced effectiveness by testing *L. lactis* NZ9803 expressing NisI and NisFEG (Table 2a) and for which a natural resistance determinant, the NSR from *S. agalactiae*, heterologously expressed by *L. lactis* NZ9000 [pNSR], no longer showed any activity (Table 2b).

Inference of structural features

Next to the bioactivity of those combinatorial peptides we were interested in the degree of post-translational modification introduced by NisB and NisC. We therefore analyzed

the final set of 11 peptides with improved activity against the pathogenic reference strains (Table 1) and the ability to bypass defense mechanisms (Table 2) by mass spectrometry. As the major modification pattern for all peptides analyzed we could identify a degree of dehydration of serine and threonine residues that is in the range of nisin (70 to 100%, nisin: 89%) and observed that except for peptide 7 (3 of 4) and peptide 10 (4 of 7) all cysteines were involved in thioether ring formation (Supplementary Figs. 10 and 11) and confirmed the success of the combinatorial design of novel lanthipeptides.

Discussion

Large-scale engineering of natural products is a promising strategy to obtain improved bioactive molecules but is suffering from two bottlenecks: a lack of insight into the determinants of functional modularity in large enzyme clusters and a lack of efficient methods to explore the antimicrobial activity of large sets of variants at high speed. Here, we show that assays for antimicrobial activity can be efficiently downscaled and parallelized, making the latter bottleneck obsolete. By switching from natural products whose structure is encoded in the reaction specificity of enzymes to those that are gene-encoded, such as lantibiotics, we drastically facilitate the production of potentially active molecule variants. We do not entirely escape the boundaries of enzyme specificity with this approach, as enzyme-based PTMs remain important, but the availability of promiscuous PTM systems as well as the sheer number of variants that DNA-manipulation can deliver in screens of the type demonstrated here, make the successful isolation of novel active, peptide-based natural products much more likely. We illustrate this by modular shuffling of lantibiotics, for which we could easily isolate 126 novel active antimicrobial peptides combining modules from other lantibiotics or of synthetic nature and some of them displaying improved or shifted activity profiles.

The presented strategy is scalable without adaptation of the protocol by increasing the number of modules, and it can readily be adapted to other pathogenic hosts[31] as well as to other library generation methods. In this study we focused on lipid II binding peptides and the nisin modification machinery. However, the strategy is applicable to all antimicrobial peptides as long as they are secreted by the producing host. We envision that the use of more diverse peptide modules, including those exhibiting a different mode-of-action, will enhance the diversity of the isolated bioactive peptides.

A bottleneck that cannot be entirely excluded is the substrate specificity of the PTM enzymes that limit the diversity of peptides that can be produced. However, given the capacity of the nanoFleming assay, future approaches might include various different PTM enzymes co-expressed in the production host or even include direct evolution on such enzyme to broaden their substrate specificity and therefore widen the diversity of the isolated antimicrobial peptides. When retaining the module shuffling strategy,

the length of the antimicrobial peptides in the library is currently restricted by the capabilities of chemical DNA synthesis (approx. 150 bases), which is sufficient for many RiPP genes, but can be extended using oligo-synthesis together with established assembly-strategies[32]. Also, the nanoFleming assay can be easily scaled to up to 10^6 clones per day, which is the current limit for large particle flow cytometry. In summary, the presented platform represents a powerful novel approach to the generation of topologically novel antimicrobial peptides.

In the future, this platform might not only be useful for the discovery of molecules with improved or altered bioactivity profiles but also for the generation of sufficient diversity required at other steps in drug development of peptides (e.g. to test candidate peptides for plasma stability or activity *in vivo*). Last, we envision the nanoFleming platform of help also for addressing other questions in peptide development, such as for the improvement of peptide secretion from host strains by genetic engineering.

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SI Methods

Chemicals and molecular biology

Unless otherwise noted, chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), DNA purification kits from Zymo Research (Irvine, CA, USA) and enzymes from NEB (Ipswich, MA, USA). Sanger-sequencing was done externally (Microsynth, Balgach, Switzerland, and GATC Biotech, Konstanz, Germany) using an appropriate primer (see Supplementary Table 3 for a list of DNA oligonucleotides). For peptide and protein purifications, IMAC equilibration buffer consisted of 500 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4; IMAC wash buffer of 20 mM imidazole, 500 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4; IMAC elution buffer I of 500 mM sodium chloride and 475 mM sodium acetate/acetic acid buffer, pH 3.5 and IMAC elution buffer II of 500 mM imidazole, 500 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4. Cleavage of leader peptides with NisPsol was done in NisP cleavage buffer containing 100 mM ammonium acetate/acetic acid, pH 6.0.

Bacterial strains and cultivations

Cloning was done using either *Escherichia coli* DH5α or *E. coli* DB3.1 (Thermo Fisher Scientific, Waltham, MA, USA, see Supplementary Table 4 for a list of strains) cultivated in 14 mL polypropylene tubes (Greiner, Kremsmünster, Austria), filled with 5 mL LBMiller broth (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 37 °C with aeration on a shaker (Kuhner, Birsfelden, Switzerland) operated at 200 rpm and 25 mm amplitude. Strain *Lactococcus lactis* NZ9000, harboring the genes for NisB, NisT and NisC on plasmid pIL3BTC (see Supplementary Table 5 for a list of plasmids) was cultivated in 14 mL polypropylene tubes filled with M17 broth (Difco) supplemented with 5 g L^{-1} glucose (GM17 broth) and incubated at 30 °C without aeration. For screening and peptide production a chemically defined medium (CDM) was used. CDM contained 83.26 mM glucose, 150.00 mM 2(Nmorpholino)ethanesulfonic acid (MES), 148.87 mM sodium chloride, 0.98 mM magnesium chloride, 20.21 µM

manganese(II) chloride, 0.24 µM ammonium molybdate, 1.07 µM cobalt(II) sulfate, 1.20 µM copper(II) sulfate, 1.04 µM zinc sulfate, 20.12 µM iron(III) chloride, 9.69 µM (±)αlipoic acid, 2.10 µM Dpantothenic acid, 8.12 µM nicotinic acid, 4.91 µM pyridoxal hydrochloride, 4.86 µM pyridoxine hydrochloride, 2.96 µM thiamine hydrochloride, 0.41 µM biotin, 1.46 mM Lalanine, 1.40 mM Larginine, 0.61 mM Lasparagine, 1.03 mM Laspartic acid, 0.35 mM Lcysteine, 0.66 mM Lglutamic acid, 0.66 mM Lglutamine, 0.39 mM glycine, 0.16 mM Lhistidine, 0.63 mM Lisoleucine, 0.89 mM Lleucine, 1.02 mM Llysine, 0.26 mM Lmethionine, 0.39 mM Lphenylalanine, 3.58 mM Lproline, 1.64 mM Lserine, 0.57 mM Lthreonine, 0.18 mM Ltryptophan, 2.76 mM Ltyrosine, 0.73 mM Lvaline. Depending on the application, CDM medium was further supplemented: CDMS (for nanoFleming screening) contained in addition 9.00 mM potassium phosphate and 7.04 mM calcium chloride and was adjusted to pH 6.5 with sodium hydroxide. CDMV (for peptide precipitation) contained in addition 20.00 mM potassium phosphate, 20.00 µM calcium chloride, and 10.00 g L^{-1} tryptone and was adjusted to pH 7.0. CDMP (for peptide purification) contained in addition 20.00 mM potassium phosphate, 20.00 µM calcium chloride, 5.00 g L^{-1} tryptone and was adjusted to pH 7.0. All media were supplemented with the appropriate antibiotics for plasmid maintenance: for *E. coli*, 20 µg mL^{-1} chloramphenicol, 250 µg mL^{-1} erythromycin, and/or 50 µg mL^{-1} kanamycin; for *L. lactis*, 10 µg mL^{-1} chloramphenicol and/or 10 µg mL^{-1} erythromycin. *Micrococcus flavus* NIZO B423 was cultivated in LBMiller broth and incubated at 30 °C with aeration. The indicator strains used in inhibition zone assays, *Staphylococcus aureus* ATCC 29213 and ATCC 33591 were cultivated in cation adjusted MuellerHinton broth (MHB 2, Difco) and incubated at 37 °C with aeration. *Enterococcus faecalis* ATCC 29212 and ATCC 51575 were incubated in ToddHewitt broth (Difco) and incubated at 37 °C with aeration. *Streptococcus pneumoniae* ATCC 49619 was cultivated in ToddHewitt broth supplemented with 10% fetal bovine serum (FBS, P303302, Milian Analytica, Rheinfelden, Switzerland) and incubated at 37 °C without aeration. The strains used for MIC testing, *S. aureus* CAL and MW2, *E. faecalis* LMG 16216, *E. faecium* LMG 16003 and *Bacillus cereus* ATCC 14579 were cultivated in MuellerHinton broth (MHB, Difco). *S. pneumoniae* TIGR4 and D39 were cultivated in MHB supplemented with 5% defibrinated sheep blood (Oxoid, Thermo Fisher Scientific). The strain *L. lactis* NZ9803 displaying nisin immunity was based on *L. lactis* NZ9800 that was genome engineered to carry a deletion of the *nisP* gene using a method described previously[33]. Expression of the immunity cluster was induced by adding 5 ng mL^{-1} nisin (from a 1 mg mL^{-1} stock in 0.05% aqueous acetic acid) to the medium. Strain *L. lactis* NZ9000 [pNSR], overexpressing the nisin resistance protein from a plasmid[30] was cultivated as described for NZ9000 but adding 5 ng mL^{-1} nisin and 10 µg mL^{-1} chloramphenicol to the medium to induce the expression of the *nsr* gene and maintain the plasmid, respectively. In case cultivations were done on solid medium, 15 g L^{-1} agar (Difco) was added to the broth.

Synthesis and cloning of the DNA library

The combinatorial peptide library was synthesized without the leader peptide sequence as DNA oligonucleotide representing the antisense strand and using solid phase synthesis (see Supplementary Figure 2) by Ella Biotech GmbH (Martinsried, Germany). It was flanked by 5' and 3' primer binding site for second strand synthesis (see Supplementary Table 6 for the DNA oligonucleotide sequences of the modules). The second strand was synthesized in a primer extension reaction using the oligonucleotide mixture and the primer *lib2ndfw* in a 1:1 stoichiometric ratio (approx. 20 pmol each in 50 µl) together with 3 units of Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) using standard PCR reaction conditions. The double stranded product was purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) and cloned into plasmid pSEVA241*silent*. The plasmid features transcriptional terminators 5' and 3' to the cloning site and it contains a *ccdB* expression cassette between NheI and HindIII cloning sites for efficient elimination of religands[34]. The plasmid was generated by amplifying the *ccdB* cassette using primers *ccdBBamHINheIfw* and *ccdBHindIIIrv* from plasmid pQL11 and then inserted into the multiple cloning site of pSEVA241. After plasmid proliferation in the CcdB resistant *E. coli* DB3.1 and Sanger-sequencing using primer *pSEVAt1fw* and *pSEVAtorv*, the library DNA was cloned into pSEVA241*silent* using *E. coli* DH5α. Growth of the transformants was done on large Petri dishes (145 mm diameter, Greiner) filled with 50 mL of LB-Miller agar and by plating approx. 1×10^4 colony forming units (CFUs) per plate (10 plates in total = approx. 15fold oversampling of the library). After incubation, the biomass was scraped off the plates and plasmid library pSEVA241*library* was isolated.

Next, the library was cloned into the screening plasmid pNZE3*rdmmcherry*. This plasmid was derived from plasmid pNZE3*nisA* and served as shuttle for transfer of the library from *E. coli* to *L. lactis*. The plasmid was first modified to add the gene for the red fluorescent protein mCherry. The gene was PCR-amplified from the BioBrick part BBa_J06504 in plasmid pSB1C3 using primers *mcherryNdeIfw* and *mcherry-HindIIIBamHIrv*. Next, the constitutive promoter P23[35] was PCR-amplified together with the RBS and the first 21 bases of the P23 regulated gene from the chromosome of *L. lactis* NZ9000 using primers *P23HindIII-NheIfw* and *P23NdeIrv*. Both PCR-products were digested with NdeI, ligated and the ligation-product was amplified using primers *P23HindIII-NheIfw* and *mcherryBamHIrv*, followed by a digest with NheI and BamHI. The same restriction sites were integrated into the plasmid pNZE3*nisA* using enzymatic inverse PCR[36] and the primers *pNZE3pNGNheIfw* and *pNZE3pNGBamHIrv*, which also contained a BsaI restriction site on both ends to circularize the plasmid. Next, the promoter::*mcherry* fusion was cloned into that plasmid and NheI site was removed using a modified QuickChange protocol[37] and primers *mcherry-NheIrmfw* and *mcherryNheIrmrv* to result in plasmid pNZE3*nisAmcherry*. Finally, the wild-type

nisA expression cassette was replaced by the cassette *nis-fragment-BglII-HindIII* containing the nisin-inducible promoter Pnis, the gene for the nisin leader peptide and the nisin structural gene (both genes were codon optimized for *L. lactis* MG1363) where a NheI restriction site was added between structural gene and the gene encoding for the leader peptide to facilitate the cloning of the leader-less library genes. The cassette was custom-synthesized (Geneart, Regensburg, Germany) and isolated from plasmid pMAT*nisAopt* by digesting with enzymes BglII and HindIII cloned into pNZE3*nisAmcherry* to result in plasmid pNZE3*nisAoptmcherry*. As a last step, the *nisA* structural gene was removed from that plasmid by digestion with NheI and HindIII and ligation of a random DNA-fragment, assembled by the annealing of the oligonucleotides *rdmNheIHindIIIfw* and *rdmNheIHindIIIrv* and resulted in plasmid pNZE3*rdmmcherry*. All pNZ-based plasmids were Sanger-sequenced using primers *pNZE3-seq-fw* or *pNZE3-seq-rv*.

The library was then transferred from the pSEVA241*library* to pNZE3*rdmmcherry* via NheI and HindIII to obtain the plasmid library pNZE3*librarymcherry* which was then used to transform strain *E. coli* DH5α. The transformants were grown on large Petri dishes filled with 50 mL LB-Miller agar supplemented with the appropriate antibiotics and by plating approx. 5×10^6 CFUs per plate (10 plates in total). The biomass was scraped off the plate and the plasmid was isolated. In the final step, the screening strain *L. lactis* NZ9000 [pIL3BTC] was transformed with the plasmid library to yield the candidate cells. The transformation mixture was again plated on large Petri dishes, this time filled with 50 mL GM17 agar, supplemented with the appropriate antibiotics and the cells were scraped off after growth for 48 h. The strain was diluted in liquid culture (GM17 broth, supplemented with appropriate antibiotics) to an OD_{600} of 0.5 and incubated at 30 °C for 3 h. The culture was then supplemented with glycerol to a volume fraction of 20%, 1 mL aliquots were frozen at 80 °C and the colony forming units (CFUs) of the stock were determined by plating.

Library quality control

The modular composition of the library was analyzed by next generation sequencing (Illumina MiSeq platform) at the following stages: (I) directly after second strand synthesis; (II) in plasmid pSEVA241*library* after library cloning in *E. coli*; (III) in plasmid pNZE3*librarymcherry* after library cloning in *E. coli*, and (IV) after transfer to *L. lactis*. The double-strand DNA fragment from stage I was directly used for sequencing. For stage II, III and IV, the library fragment was isolated from the plasmids by NheI and HindIII digestion followed by purification with an agarose gel. The linear DNA fragments were processed as recommended by the MiSeq Reagent Kit and sequenced in a paired-end run with 251 cycles on a MiSeq device (Illumina, San Diego, CA, USA) running RTA, version 1.18.54 (provided by the device manufacturer). Raw data were

processed using the software bcl2fastq, version 2.18.0.12 (provided by the device manufacturer) and the resulting FASTQ files from each sequencing run were processed using an in house written software to identify module counts, sequence mismatches and indels. The resulting datasets were used to judge bias and error rate of each of the synthesis and cloning steps (see Supplementary Figure 2).

nanoFleming screening

Depending on the experiment, the candidate strain either carried a plasmid for secretion of prenisin (pNZE3*nisAmcherry* = positive control), an empty plasmid (pNZE3*rdmmcherry* = negative control) or the plasmid library (pNZE3*librarymcherry*). As sensor strain, *M. flavus* (NIZO B423) or *L. lactis* NZ9000 [pNG*nisTPtdgfp*] was used. Encapsulation of cells into nLRs was done as previously described[24] using laminar-jet breakup (VAR D encapsulator from Nisco Engineering, Zürich, Switzerland) of a sodium alginate solution (20 g L^{-1} alginate in water, sterile-filtered) and using bacterial glycerol stocks. CFUs were adjusted such that on average each nLR contained 0.3 candidate cells and 150 sensor cells. The encapsulator was operated at 0.7 kHz with a flow rate of 3.3 mL min^{-1} and a nozzle diameter of 150 µm. Reactors were solidified in nLR hardening buffer (1 mM tris(hydroxymethyl)aminomethane hydrochloride (TrisHCl) pH 7.0, 100 mM $CaCl_2$) for 20 min, and briefly rinsed with nLR wash buffer (1 mM TrisHCl pH 7.0, 10 mM $CaCl_2$). The average nLR diameter after hardening was 460 µm (approx. 50 nL, CV: 4 to 7%). The nLRs where then transferred to CDMS medium (100 g nLR L^{-1} and incubated for 6 h at 30 °C on a shaker (200 rpm, 25 mm amplitude, Kuhner, Birsfelden, Switzerland). The nutrients provided in this medium are not sufficient for growth of the sensor but allow for growth of the candidate (candidate head start). After 6 h, the medium was supplemented with 5 ng mL^{-1} nisin to induce peptide production. After an additional hour of incubation, 10 g L^{-1} tryptone was added (from a 100 g L^{-1} stock in water) to allow for the growth of the sensor. Furthermore, the protease NisPsol was added (only if not already expressed by the sensor strain) at a final concentration of 0.2 µg mL^{-1} (from a 20 µg mL^{-1} enzyme stock in 100 mM MES buffer, pH 6.0). The nLRs were incubated for another hour and then removed from the medium using a cell strainer (100 µm mesh size, Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) and an aliquot of approx. 1 g (wet weight, approx. 2×10^4 nLRs) was added to a 50 mL centrifugation tube pre-filled with 45 mL of a hydrophobic phase (mineral oil heavy, Carl Roth, Karlsruhe, Germany), supplemented with surfactants (40 g L^{-1} Span80 and 10 g L^{-1} Tween85). Emulsification was achieved by vigorous shaking, the whole content of the tube was poured into a large Petri dish (145 mm diameter) and incubated at 30 °C for 18 h.

After incubation, the emulsion was transferred into a sterile glass beaker and the oil was decanted. The nLRs were then transferred to 50 mL centrifugation tubes (approx.

10 mL of wet nLRs per tube) and washed several times with nLR wash buffer supplemented with 0.1 g L^{-1} Tween20, followed by centrifugation (1,000x g, 1 min) until all remaining oil was removed. The nLRs were then transferred to 50 mL of fresh nLR wash buffer and the biomass was stained (if not labeled by tdGFP) by the addition of 1 µM of SYTO 9 (from a 5 mM stock in DMSO, Thermo Fisher Scientific) followed by incubation in the dark for 1 h at room temperature at approx. 20 rpm on a benchtop roller incubator.

The nLRs were then analyzed using a large-particle flow-cytometer (BioSorter, Union Biometrica, Holliston, MA)[23]. The device was operated with water as sheath fluid and analysis was done with extinction at 488 nm as a trigger signal and recording data for time-of-flight (TOF, as a relative estimate of the particle size), extinction at 488 nm, green fluorescence (sensor, excitation laser 488 nm, beam splitter DM 562, emission filter BP 510/23 nm) and red fluorescence (candidate, excitation laser 561 nm, TR mirror, emission filter BP 615/24 nm). Signal analysis and selection of subpopulations was done using the Flow Pilot software, version 1.3.08 (provided by the device manufacturer). Data analysis was done using the FlowJo software, version 10.1 (FlowJo LLC, Ashland, OR, USA). Prior analysis of the library, samples containing nLR with embedded colonies of the positive control and negative control were analyzed. For library screening, nLRs displaying low green fluorescence intensity levels (lower than the mean green intensity of the negative control by at least 3 σ) were sorted into a 50 mL centrifugation tube prefilled with 10 mL nLR wash-buffer (device specific 'enrichment mode', max. 150 Hz sorting rate). Then, isolated nLRs were subjected to another sorting ('pure mode', max. 1 Hz sorting rate), this time spotted into Nunc MicroWell 96well plates (167008, Thermo Fisher Scientific) filled with 100 µL of GM17 broth which was supplemented with 10 µg mL^{-1} chloramphenicol and 10 µg mL^{-1} erythromycin to allow for selective recovery of the candidate strain while killing the sensor strain. The plates were sealed (airtight aluminum foil) and incubated at 30 °C for 72 h without shaking to expand the candidate strain from the nLR. Cultures in the wells were then supplemented with glycerol to a final volume fraction of 20%, the plates were sealed and frozen at 80 °C.

Fluorescence microscopy

Microscopic analysis of nLRs was carried out with the inverted fluorescence microscope Axio Observer II equipped with an AxioCam MR3 camera (Carl Zeiss Microscopy, Jena, Germany) either using bright-field or epifluorescence with filter cubes (for GFP, SYTO 9: excitation BP 470/40 nm, beam splitter DM 495 nm, emission BP 525/50 nm; for mCherry: excitation BP 565/30 nm, beam splitter DM 585 nm, emission BP 620/60 nm). Images were taken using the AxioVision software, version 4.8.2 SP3 (provided by the device manufacturer). If bright-field and epifluorescence were recorded from the same object, images were stored as overlays of both channels. Image processing and analysis was done using the Fiji software[38,39].

Peptide identification and precipitation

For each peptide, a culture in 10 mL GM17 broth inoculated from a single colony of the peptide producing strain was prepared. After growth, 6 mL of the culture was used to isolate the plasmid and the peptide gene was Sanger-sequenced using primer *pNZE3seqfw*. Next, the peptide was precipitated by trichloroacetic acid (TCA)[40]. For this, 90 mL of CDM-V medium supplemented with the appropriate antibiotics and 5 ng mL^{-1} nisin was inoculated with 900 µL from the GM17 culture. After 24 h of incubation, the cells were pelleted by centrifugation (3,200× g, 30 min). The peptide was precipitated by adding 10 mL of an ice-cold, saturated trichloroacetic acid solution (in water) to the supernatant and freezing (−20 °C) the mixture for >2 h. After thawing, the precipitated peptide was pelleted by centrifugation (48,000× g, 30 min 4 °C) and washed once with ice-cold acetone followed by a second centrifugation. The pellet was dried at room temperature and resuspended in 1 mL of an 0.05% aqueous acetic acid solution. Next, the leader peptide was cleaved off using 750 µL of the peptide solution, 250 µL of 4× NisP cleavage buffer and NisPsol at a final concentration of 0.2 µg mL^{-1} (from a 20 µg mL^{-1} enzyme stock). The mixture was incubated at 37 °C until complete cleavage was achieved (approx. 36 h, monitored by HPLCMSMS, see below). Each peptide was precipitated in duplicate.

Leader peptide quantification

The amount of leader peptide in the NisPsol treated peptide samples was quantified by HPLCMSMS using an Agilent 1200 series HPLC system coupled to an Ab Sciex 4000 QTRAP triple quadrupole mass spectrometer (operated with Analyst software, version 1.6.3, Ab Sciex, Framingham, MA) and using electrospray ionization (ESI). An aliquot of 3 µL of the peptide sample was injected onto a RPC18 column (ReproSil-Pur Basic C18 3 µm, 50 × 3 mm, Dr. Maisch, Ammerbuch, Germany), heated to 30 °C and operated with water supplemented with 0.1% formic acid as solvent A and acetonitrile supplemented with 0.1% formic acid as solvent B (all solvents MSgrade). The column was equilibrated at 10% solvent B prior injection. After injection, a gradient was imposed from 10% solvent B to 35% solvent B in 180 s at a flow rate of 800 µL min^{-1}. The column was washed with 95% solvent B for 45 s at 1,500 µL min^{-1} and equilibrated with 10% solvent B for 60 s at 1,500 µL min^{-1}. Usually three leader peptide peaks were observed: peptide 1, without methionine, resulting from cleavage by endogenous methionine aminopeptidases; peptide 2, with a non-formylated methionine at the N-terminus; peptide 3, with a formylated methionine at the N-terminus. For quantification of the leader peptides, the mass spectrometer was operated in multiple reaction monitoring (MRM) mode. The parameters for the TurboIonSpray probe: ion spray voltage (IS): 5,000 V, positive polarity, temperature (TEM): 700 °C, curtain gas (CUR): 20 psig, ion source gas 1 (GS1): 70 psig, ion source gas 2 (GS2): 60 psig, interface heater (ihe): ON and

the settings for the MS: declustering potential (DP): 80 V, entrance potential (EP): 10 V, collision energy (CE): 45 V, collision cell exit potential (CXP): 15 V and collision gas (CAD): 4 psig. Quantification was done for the fragment ion (Q3) at 574.4 m/z originating from the mother ions (Q1) from leader peptide 1 at 589.4, 784.9, 1,176.6 m/z, from leader peptide 2 at 622.0, 829.0, 1,242.5 m/z and from leader peptide 3 at 629.0, 838.0, 1,256.5 m/z with 50 ms dwell time. Signal peaks were integrated using Analyst software, version 1.6.3 (provided by the device manufacturer) and the sum of the peaks was normalized to the leader amount that resulted from a precipitated prenisin control (relative quantification). Measurements were performed in triplicate.

Inhibition zone assay

The antimicrobial activities of the precipitated peptides were confirmed using an inhibition zone assay[28]. The strains *M. flavus* (NIZO B423), *S. aureus* MSSA (ATCC 29213) and MRSA (ATCC 33591), *E. faecalis* VSE (ATCC 29212) and VRE (ATCC 51575) and *S. pneumoniae* (ATCC 49619) were used as sensor strains. Plates were assembled using 50 mL of the strain-specific agar, cooled to 40 °C and mixed with 2 mL of a culture at an OD_{600} of approx. 1. The mixture was poured into large Petri dishes (145 mm diameter) and cooled to room temperature. Using a stamp, 19 holes (3 mm diameter) were inserted into the agar and 50 µL of the samples were pipetted into each hole. Plates were incubated for 24 h and imaged using a flatbed scanner. Zone areas were measured using the Fiji software[38,39]. Inhibition zone areas were normalized to areas that resulted from a precipitated nisin control (relative quantification). Measurements were performed in triplicate.

Peptide production and purification

The leader peptide of each variant was equipped with a His_6tag followed by an additional tryptophan (HWtag, see Supplementary Table 7 for DNA and peptide sequences). The tag was integrated by enzymatic inverse PCR using primers *tagHisWBsaIfw* and *tagHisWBsaIrv*, the product was cleaved with BsaI and re-circularized by ligation. The mix was used to transform *L. lactis* NZ9000 [pIL3BTC]. The integration of the tag was confirmed by Sanger-sequencing using primer *pNZE3seqfw*.

For peptide purification, a 25 mL CDMP preculture, supplemented with the appropriate antibiotics, of the peptide producing strain was incubated overnight. Then, 20 mL of the culture was used to inoculate 2 L of CDMP medium supplemented with the same antibiotics and 10 ng mL^{-1} nisin. The culture was incubated without shaking until it reached stationary phase (after 24 to 36 h). After incubation, the cells were pelleted by centrifugation (6,000× g, 30 min). The pH of the supernatant was adjusted to 7.0, filtered through a bottle-top filter (0.22 µm pore size, PES membrane, Stericup, Merck Millipore, Billerica, MA, USA) and stored at 4 °C. The pellet was resuspended in

50 mL of 70% isopropanol, 0.4% TFA and stirred at room temperature for 2 h to separate cell-bound peptides from pellet components. After centrifugation (3,200× g, 10 min), the cell pellet was discarded and the isopropanol in the supernatant was removed with a rotary evaporator at 40 °C and 40 mbar for 10 min. The pH of the remaining solution was adjusted to 7.0 with NaOH and added to the previously retained supernatant for further treatment on an ÄKTAexplorer chromatography system (operated with Unicorn software version 5.31, GE Healthcare, Chicago, IL). The system was connected to a NiSepharose EXCEL column (XK 16/20, 10 mL bed volume, GE Healthcare) equilibrated with 5 column volumes (CV) of IMAC equilibration buffer. The culture supernatant was loaded onto the column at a flow rate of 10 mL min^1. The column was washed with 10 CV of IMAC equilibration buffer. The peptides were eluted with 3 CV of IMAC elution buffer I followed by 3 CV of IMAC elution buffer II. The whole elution fraction (60 mL) was collected and then loaded onto a Sephadex G15 column (XK 50/30, 300 mL bed volume) for desalting that was equilibrated with 5 CV of desalting buffer pH 4.0 (100 mM ammonium acetate/acetic acid buffer, pH 4). The same buffer was used for elution. The elution fraction corresponding to the peptide (monitored at 280 nm absorbance) was collected (approximately 100 mL), frozen at −80 °C for >2 h and lyophilized (approx. 60 h) using a freeze-dryer (Alpha 12 LDplus, Christ, Osterode, Germany), connected to a vacuum pump (RC6, Vacuubrand, Wertheim, Germany).

To remove the leader peptide, the freeze-dried peptides were dissolved in 40 mL of NisP cleavage buffer containing NisPsol at a concentration of 0.2 µg mL^{-1} and incubated at 37 °C for 16 h. Cleavage of the leader peptide was monitored by HPLC (see below). When incomplete, more NisPsol was added. For HPLC analysis an aliquot of 3 µL of the peptide was injected onto an RPC18 column (ReproSil-Pur 120 C18-AQ 3 µm, 150 × 2 mm, 5 × 2 mm precolumn, Dr. Maisch) heated to 30 °C and operated at a flow rate of 300 µL min^{-1} with water supplemented with 0.1% TFA as solvent A and acetonitrile supplemented with 0.1% TFA as solvent B (all solvents MSgrade). The column was equilibrated with 20% solvent B prior injection. After injection and an initial wash step of 2.8 min a gradient was imposed from 20% solvent B to 50% solvent B in 16 min. The column was washed with 95% solvent B for 5 min and equilibrated with 20% solvent B for 9.2 min. Peptide elution was monitored at an absorbance of 205, 254 and 280 nm.

HPLC-purification of the cleaved peptides was performed on an ÄKTAexplorer chromatography system. The complete peptide sample was injected onto a RPC18 column (PRONTOSIL 120 C18 10 µm, 250 × 20 mm, 50 × 20 mm precolumn, Bischoff, Leonberg, Germany), heated to 30 °C and operated at a flow rate of 10 mL min^{-1} and with water supplemented with 0.1% TFA as solvent A and acetonitrile supplemented with 0.1% TFA as solvent B. The column was equilibrated with 20% solvent B prior injection. After injection and an initial wash step of 6 min a gradient was imposed from 20% solvent B to 50% solvent B in 40 min. The column was washed with 95%

solvent B for 8 min and equilibrated with 20% solvent B for 13 min. Peptide elution was monitored at an absorbance of 205 nm and peptide peaks were collected. The fractions were frozen at −80 °C for >2 h and lyophilized (approx. 18 h) using a freeze-dryer (Alpha 2-4 LDplus, Christ), connected to a vacuum pump (RC6, Vacuubrand). In case where a peptide resulted in several peaks (e.g. due to different PTM patterns of the same translation product), all peaks were MIC-tested (see below) but only the peak with the highest activity was processed further.

NisP production

The protease was secreted as soluble form (NisPsol) where the membrane anchor of the enzyme was replaced by a His_8-tag and expressed from plasmid pNZ*nisPsol*-8H. For production, a 10 mL GM17 preculture, supplemented with the appropriate antibiotics, of the strain was used to inoculate a 1 L production culture in GM17 broth, supplemented with the same antibiotic. At an OD_{600} of 0.7, 5 ng mL^{-1} nisin was added and incubation was continued for 18 h and the culture was processed as described for the peptide purification. Purification was done on an ÄKTAexplorer chromatography system and using a Sepharose 6 Fast Flow column (GE Healthcare), loaded with Co^{2+} ions (XK 16/20, 10 mL bed volume) equilibrated with 5 CV of IMAC equilibration buffer. The supernatant was loaded onto the column at a flow rate of 5 mL min^{-1}. The column was washed with 10 CV of IMAC equilibration buffer. The protein was eluted with 6 CV of elution buffer II and fractions containing NisPsol were pooled (approx. 30 mL) and then loaded onto a Sephadex G15 column (XK 50/30, 300 mL bed volume) for desalting using a buffer containing 100 mM MES at pH 6.5. Next, glycerol was added to a final volume fraction of 10%, the protein amount was adjusted to 20 µg mL^{-1} and the protease was frozen at −80 °C.

If NisP was produced as membrane-bound variant (e.g. for *L. lactis* as sensor strain), plasmid pNG-*nisTP-gfp* was used. The plasmid is based on pNG-*nisTP* that was modified to carry the gene for tdGFP. The modification was done as described for pNZE3-*nisA-mcherry* using primers *pNZE3pNG-NheI-fw* and *pNZE3pNG-BamHI-rv* for enzymatic inverse PCR and cloning the gene for tdGFP from plasmid pKQV5-*tdgfp* using primers *tdgfp-NdeI-fw* and *tdgfp-HindIII-BamHI-rv*.

Measurement of MICs

For MIC measurements, the HPLC-purified and lyophilized peptides were resuspended in 1 mL of an 0.05% aqueous acetic acid solution and analyzed by HPLC as described above. The concentration was measured using the area under the curve at 205 nm and peptide-specific absorption properties[41,42]. For MIC assays, the bacteria were grown overnight on strain-specific agar plates. The peptides were diluted with 0.05% acetic acid to a concentration of 128 to 1024 µg mL^{-1} (depending on the strain tested). The

exact amount of peptide in the dilution was again quantified via HPLC. MICs were measured as microdilution assay in 96-well flat bottom polypropylene plates (Greiner) and performed as described by Wiegand et al.[43]. The plates were sealed (airtight aluminum foil) and incubated for 18 h without shaking at the strain-specific temperature before reading the OD_{600} using an Infinite Pro F200 plate reader (Tecan, Männedorf, Switzerland). The MIC value was set as the minimal concentration where no growth of the bacterial strain was observed (<5% of the OD_{600} value of the positive growth control in column 11). MIC experiments were performed in triplicate.

Mass-spectrometric characterization of peptides

Each peptide was analyzed twice, first to determine the number of dehydrations and second to quantify the sulfhydryl groups. The difference between the two was then used to elucidate the number of thioether-rings that must have been formed. Dehydrations were characterized by mass shifts of −18 Da, sulfhydryls were measured indirectly by derivatization of those groups with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) resulting in a mass shift of +25 Da. For analysis, the HPLC-purified and lyophilized peptides were resuspended in 0.05% aqueous acetic acid to reach a concentration of 1 to 5 mg mL^{-1}. Next, 25 µL of the peptide was mixed with 1 µL of a 500 mM tris(2-carboxyethyl)phosphine solution (TCEP, in water, freshly prepared) in HPLC glass vials (>10-fold molar excess of TCEP) and incubated for 10 min at room temperature. For quantification of dehydrations, 24 µL of an 0.05% aqueous acetic acid solution was added and the sample was immediately measured. For quantification of sulfhydryls, 24 µL of a 178 mM CDAP (in 0.05% aqueous acetic acid, freshly prepared) was added (>100-fold molar excess of CDAP) and incubated for 30 min at room temperature prior measurement.

Measurements were performed using an Agilent 1200 series HPLC system coupled to an Ab Sciex 4000 QTRAP triple quadrupole mass spectrometer and using electrospray ionization (ESI). The device was calibrated prior measurement using the MS Chemical Kit 1, Low-High Conc. PPGs (Ab Sciex, 4406127) according to the manufacturer's recommendation. An aliquot of 2 µL of the sample was injected onto an RPC18 column (ReproSil-Pur 120 C18-AQ 3 µm, 150 × 2 mm, 5 × 2 mm precolumn, Dr. Maisch) heated to 30 °C. The HPLC parameters were the same as for the peptide quantification (see above) but using formic acid instead of TFA. The mass spectrometer was operated in Q1 mode. The parameters for the TurboIonSpray probe: ion spray voltage (IS): 5,000 V, positive polarity, temperature (TEM): 300 °C, curtain gas (CUR): 20 psig, ion source gas 1 (GS1): 70 psig, ion source gas 2 (GS2): 60 psig, interface heater (ihe): ON and the settings for the MS: declustering potential (DP): 60 V, entrance potential (EP): 10 V, collision cell rod offset (RO2): −60 V, Q3 rod offset (RO3): −62 V and exit lens (EX): −75 V.

Each sample was analyzed twice, a first explorative scan from 500 to 2,000 m z^{-1} at a scan rate of 1,000 Da s^{-1} was followed by a precise scan covering a window of 100 m z^{-1} at a scan rate of 250 Da s^{-1} for a maximum of three charge states.

The data was processed using a script written in the programming language R and employing the MALDIquant package[44]. The data was deconvoluted, averaged and centroided (signal-to-noise threshold: 5%). The results where then compared to the calculated peptide masses[41] to determine the number of dehydrations or sulfhydryl groups.

Statistical analysis

For nanoFleming screening, statistical parameters consisted of the absolute number of nLRs analyzed, the numbers of nLRs within the low and high green fluorescence subpopulations as well as the mean green fluorescence, its standard derivation (SD) and coefficient of variation (CV). All parameters were calculated using the Flow Pilot software (version 1.3.08, Union Biometrica) directly during screening. For MIC measurements, only those experiments were considered as reproducible where the spread of the three MIC values defined as $\log_2$(MICmax - MICmin) was ≤2. The statistical evaluation of the MIC values was done with a script written in the programming language R.

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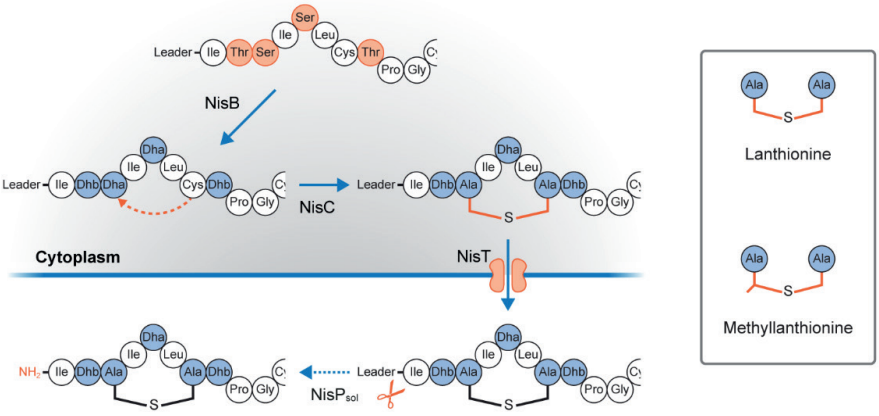
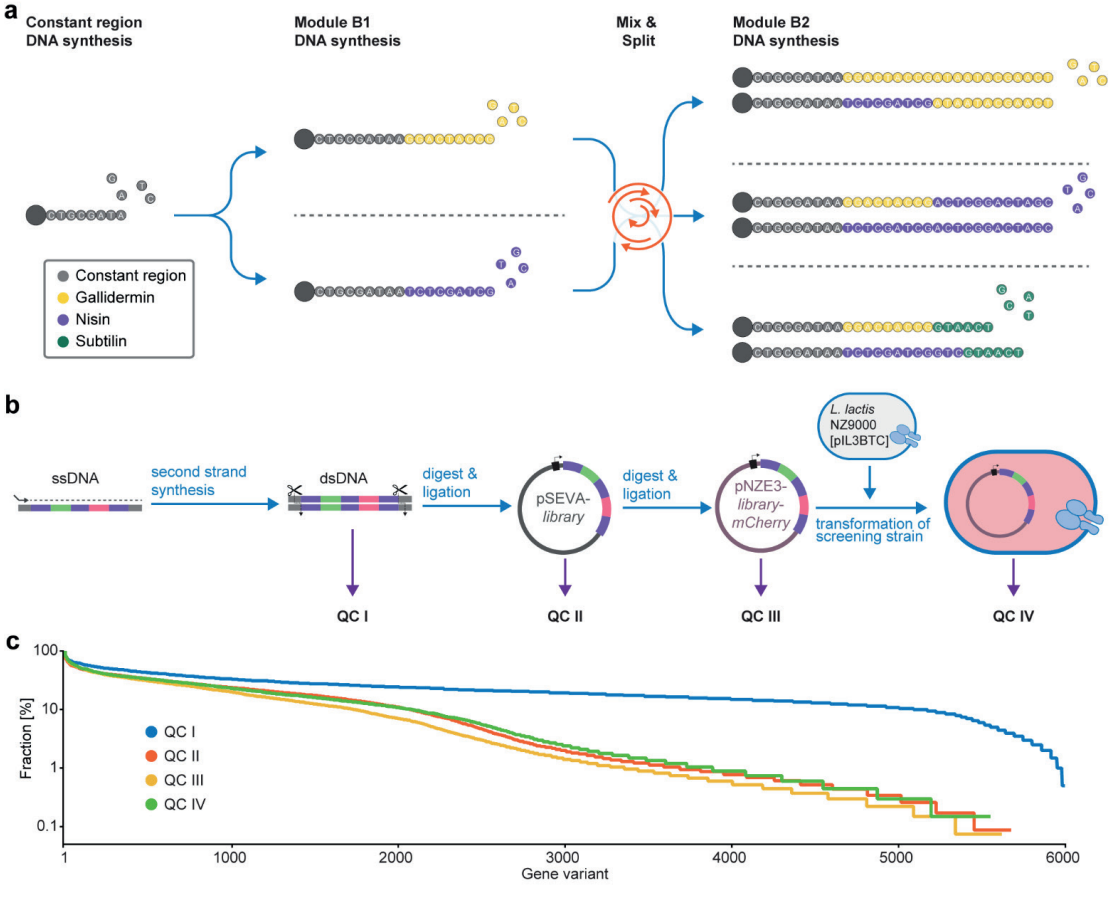
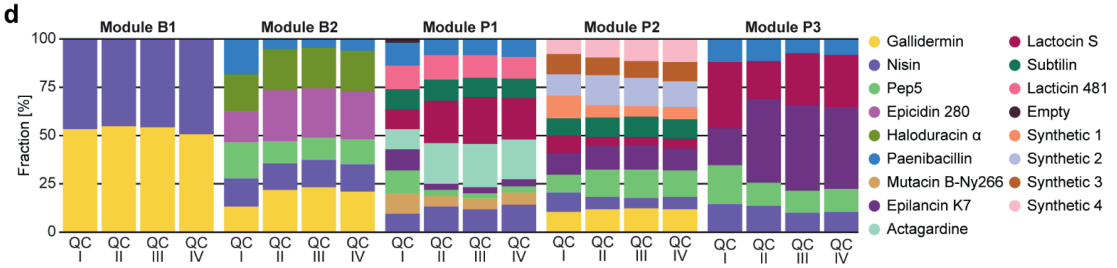
Supplementary Figures and Tables

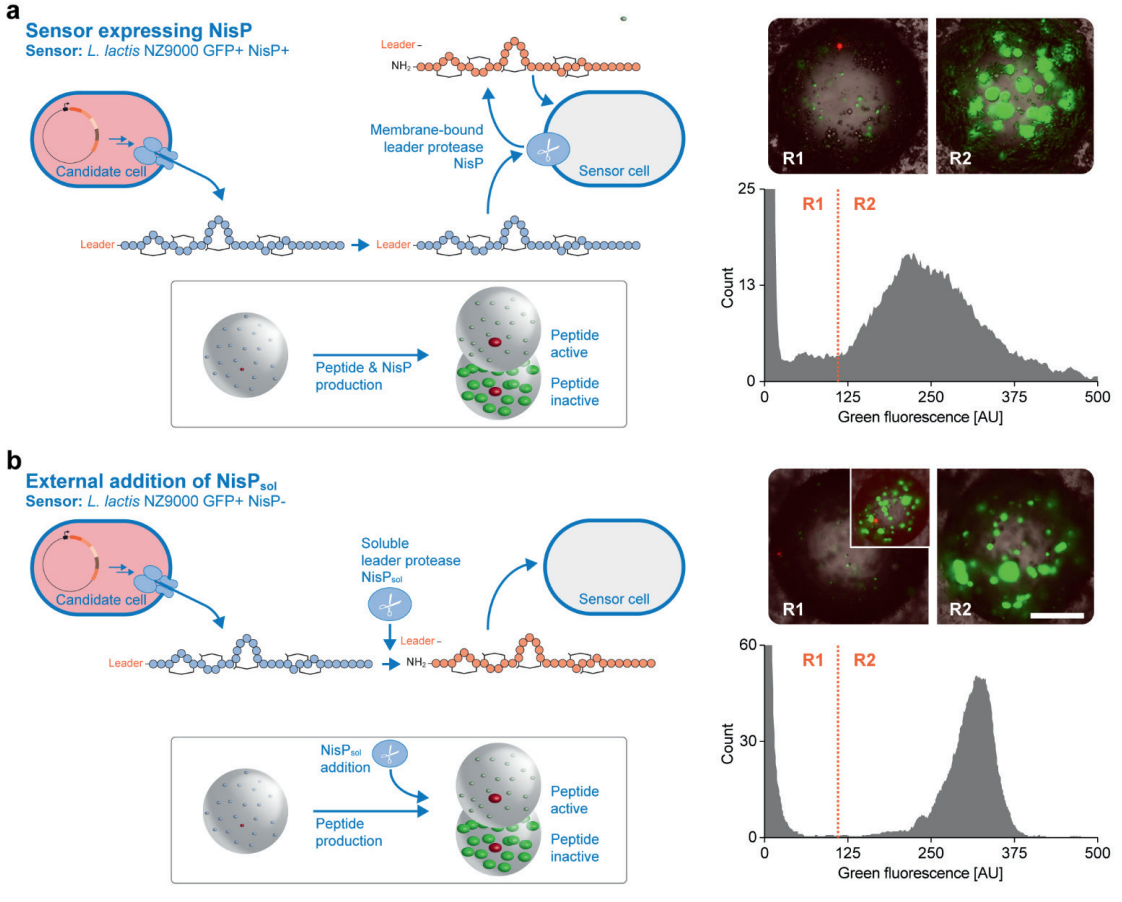


Supplementary Figure 1. Post-translational modification machinery. Four different enzymes (NisB, NisC, NisT, and NisP) were used for post-translational processing and secretion of the combinatorial peptides. Furthermore, to direct the non-modified precursor peptide synthesized at the ribosome to the modification machinery, the peptides were fused to the leader peptide of nisin. After ribosomal synthesis, the dehydratase NisB dehydrates serine and threonine residues to dehydroalanine or dehydrobutyrine, respectively. Next, the cyclase NisC catalyzes ring formation via a nucleophilic attack of a cysteine residue to the dehydroamino acids following a Michael addition type reaction mechanism leading to the formation of lanthionine (after dehydration of serine) or methyllanthionine (after dehydration of threonine). Next, NisT secretes the peptide to the extracellular space. For activation, the leader-specific protease NisP cleaves the leader peptide to release the active core peptide. Note that while wild-type NisP is membrane-bound, we used a soluble, not membrane associated variant of NisP (NisPsol) instead.

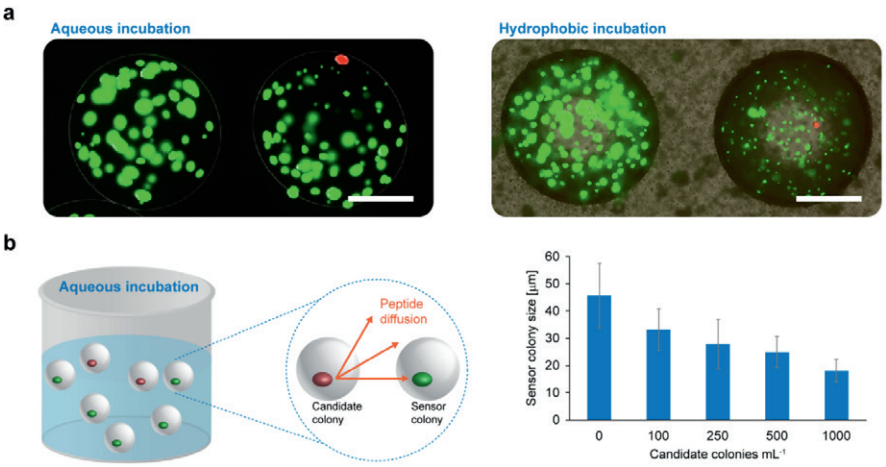

a
Constant region DNA synthesis
Module B1 DNA synthesis
Mix & Split
Module B2 DNA synthesis
Constant region
Gallidermin
Nisin
Subtilin
b
ssDNA
second strand synthesis
dsDNA
digest & ligation
pSEVA-library
digest & ligation
pNZE3-library-mCherry
L. lactis NZ9000 [pIL3BTC]
transformation of screening strain
QC I
QC II
QC III
QC IV
c
Fraction [%]
100
10
1
0.1
QC I
QC II
QC III
QC IV
1
1000
2000
3000
4000
5000
6000
Gene variant



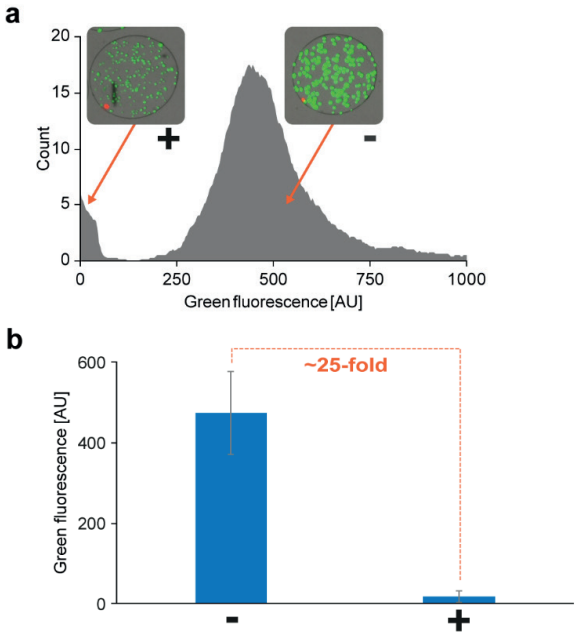
Supplementary Figure 2. Synthesis, cloning and quality control of the combinatorial library. (a) The library was generated using chemical solid phase synthesis on controlled pore glass (CPG) combined with a mix-and-split approach. We performed DNA synthesis of the antisense/minus strand of the combinatorial genes in one pot. The synthesis starts with the first module, a conserved region among the whole library (grey). This region contains restriction sites later used for cloning. After the synthesis of this constant region, the synthesis batch is, while still bound to the CPG, split up and the oligonucleotide is elongated by the synthesis of the variants of module position B1 in separate batches (yellow and violet, corresponding to the first modules of the library, gallidermin and nisin). Next the batches are pooled, mixed and separated again for the synthesis of module position B2 (yellow, violet, green). The procedure is continued until the last module is reached - which is again a constant region facilitating cloning. (b) The cloning of the combinatorial library involves several steps. First, the oligonucleotide is released from the CPG and the second strand (sense/plus strand) of the library is synthesized using an oligonucleotide complementary to the 3' constant region. After that, the double strand DNA is digested using restriction enzymes, ligated in a subcloning plasmid and proliferated in *E. coli*. The amplified plasmid is then isolated and the library fragments are excised via restriction digest, cloned into the expression plasmid and proliferated in *E. coli* again. In a last step, the plasmid is isolated from *E. coli* and transferred into the screening host *L. lactis* NZ9000 [pIL3BTC]. Every step of the protocol can result in an introduction of errors in the library (such as a shift in the modular distribution or in the accumulation of mutations). We therefore included quality control (QC) of the library composition at every processing step (indicated as QC I to IV). (c) The quality of the library is assessed via next generation sequencing with an Illumina MiSeq platform. After sequencing, every possible module combination is counted, normalized to the variant with the highest abundance and plotted. Whereas for the double stranded oligonucleotide at QC I 5,997 of 6,000 (99.95%) module combinations were found at least once for the library (NGS coverage of 44-fold), the last cloning step and after the transfer to *L. lactis* in QC IV yielded in 5,552 of 6,000 (92.5%) module combinations with counts >0 (NGS coverage of 68-fold). Moreover, the results indicate a shift in the distributions of the module combinations towards the under- and overrepresentation of certain variants. (d) Next, the distribution for each module was extracted. Whereas in QC I each module position seems to be evenly occupied by every possible module (except the 'empty' module at P1), during the first cloning step and detected at QC II, the modular composition shifts such that certain module combinations are under- and overrepresented. However, still all modules were detected and are present in the library and therefore qualifies the library for screening.


a
Sensor expressing NisP
Sensor: L. lactis NZ9000 GFP+ NisP+
Leader-
NH2
Membrane-bound
leader protease
NisP
Candidate cell
Sensor cell
Leader
Leader
Peptide & NisP
production
Peptide
active
Peptide
inactive
R1
R2
25
13
0
Count
0
125
250
375
500
Green fluorescence [AU]
b
External addition of NisPsol
Sensor: L. lactis NZ9000 GFP+ NisP-
Soluble
leader protease
NisPsol
Candidate cell
Sensor cell
Leader
Leader-
NH2
NisPsol
addition
Peptide
production
Peptide
active
Peptide
inactive
R1
R2
60
30
0
Count
0
125
250
375
500
Green fluorescence [AU]

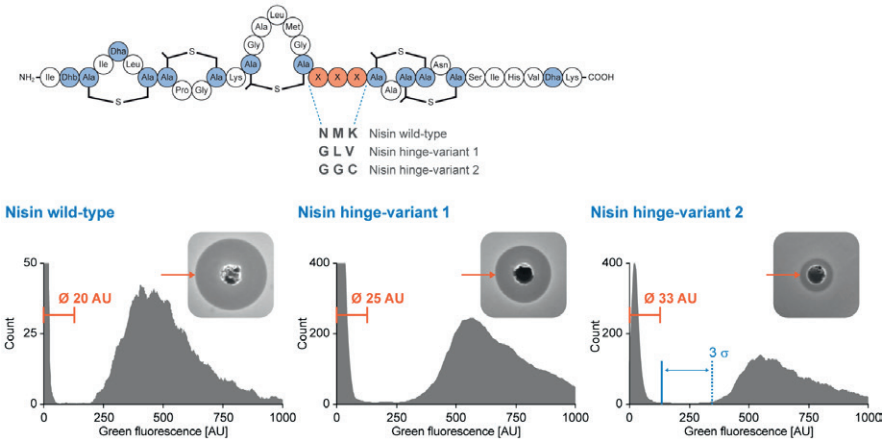
Supplementary Figure 3. Peptide activation strategies in nLRs. The combinatorial peptides are produced and secreted bound to a leader peptide and thus still inactive. Upon secretion, the peptides diffuse within the matrix of the nLR. In order to assess their antimicrobial potency, the leader peptide has to be cleaved off. Two strategies were selected as most sui (a) The leader-specific protease NisP is heterologously produced by the sensor strain (e.g. *L. lactis* NZ9000 [pNG*nisTPtdgfp*]) and bound to the (outer) membrane of the sensor cells. Here, NisP can activate the peptides once they reach the sensor and the activated peptides can then inhibit the growth of the sensor cells. The successful implementation of this activation was shown by microscopy (images are overlays of bright-field and epifluorescence images using GFP and mCherry filter sets). The left image represents a nLR (still in the hydrophobic phase) with a prenisin producing colony (red). Reduction of green fluorescence when compared to the candidate-free nLR on the right indicates growth inhibition of the GFP-labeled sensor cells due to secretion of an active antimicrobial peptide. The same result is observed when nLRs are analyzed by large-particle flow cytometry: a separation between the two nLR-types can be achieved (R1 corresponds to nLR where an active compound is produced and R2 to candidate-free nLRs). (b) Here, a *L. lactis* strain without the gene for the leader protease NisP is used as sensor. NisP is then added as soluble enzyme from the outside of the nLR and prior incubation in the hydrophobic phase. Similar to a, the activation strategy works and leads to growth inhibition of the sensor strain in the case where a prenisin-secreting colony is present. Note that when no NisP is added to the nLR, the secreted peptide cannot be activated and no sensor growth inhibition is observed (small inset). When analyzed by large-particle flow cytometry, the separation is even more clear than in the case of heterologously expressed NisP, possibly because of the amount of protease present directly from the beginning of the incubation is higher in cases in which the protease is co-embedded rather than expressed from the sensor cells. Furthermore, the expression of *nisP* might have a negative impact on the growth of the sensor-cells and further decreases the separation of both populations. Scale bar: 200 µm.



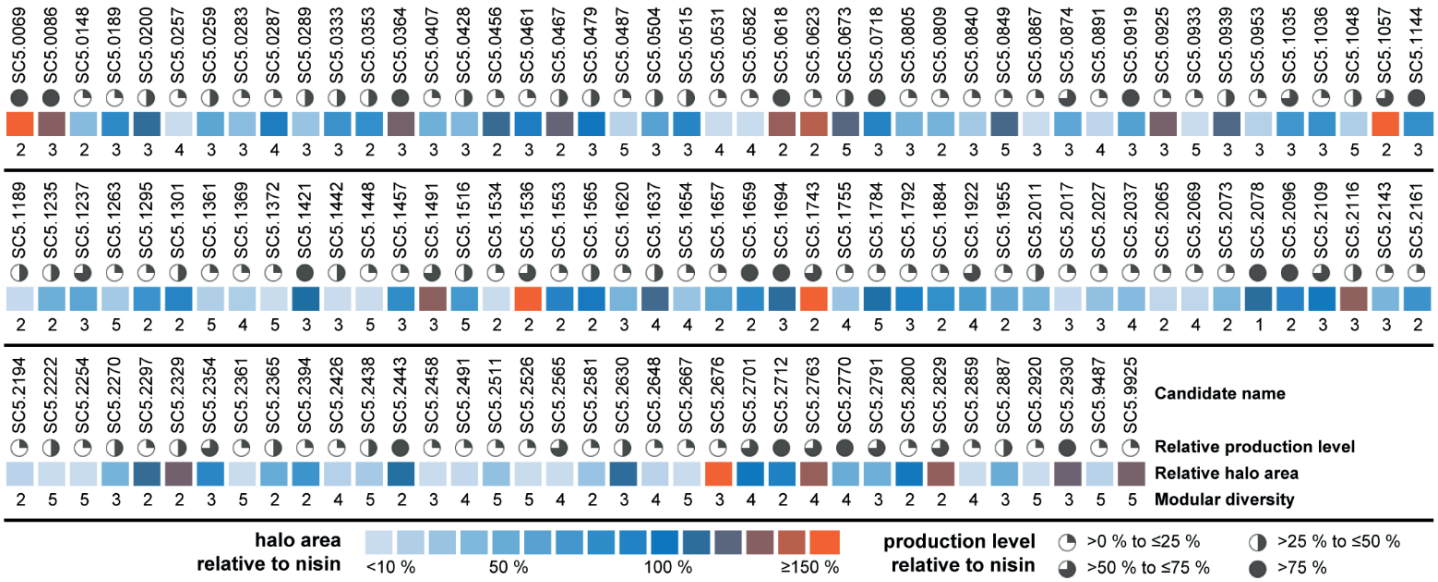
Supplementary Figure 4. Diffusion and crosstalk of nisin among nLRs. (a) Two nLR incubation methods were compared. First, the incubation of nLRs was solely done in aqueous phase (CDMS growth medium). Peptides secreted by the candidate strain *L. lactis* NZ9000 [pIL3BTC, pNZE3*nisAmcherry*] can therefore diffuse not only inside the nLR but eventually also leave the nLR which might lead to incomplete inhibition of the sensor growth inside the nLR as shown by microscopic analysis (left picture, images are overlays of bright-field and epifluorescence images using the GFP and mCherry filter set). Note the gradient of the sensor growth in the right nLR. In contrast, if incubation is performed in a hydrophobic phase (right picture), growth inhibition of the sensor is complete throughout the nLR. Scale bars: 200 µm. (b) To test whether the diffusion of peptides out of the reactors during aqueous incubation can also lead to an inhibition of cells in other nLRs in the same incubation batch we mixed nLRs containing either colonies of the candidate strain *L. lactis* NZ9000 [pIL3BTC, pNZE3-*nisA-mcherry*] or colonies of the NisP-producing sensor strain *L. lactis* NZ9000 [pNG*nisTPtdgfp*] (on average 1 colony nLR^{1}, Poisson diluted). The nLRs were then mixed in separated batches (2,000 nLR mL^{-1} each) in fixed ratios with increasing amounts of candidate-containing nLRs (0, 100, 250, 500, 1,000 nLR mL^{-1}) and decreasing numbers of sensor-containing nLRs (2,000, 1,900, 1,750, 1,500, 1,000 nLR mL^{-1}). After mixing of the nLRs in CDMS, peptide-production was induced with 5 ng mL^{-1} nisin and the nLRs where incubated for 18 h. After incubation, nLRs where recovered from the medium and the size of the sensor colonies was measured. We could observe a decreasing colony size of the sensor colonies when increasing amounts of candidate colonies were co-incubated with the sensors. This indicates a crosstalk between the nLRs allowing a diffusion of the peptides from the 'candidate' nLRs to the 'sensor' nLRs and thus inhibiting the growth of those cells. Bars represent the mean of 50 colonies (n = 50), error bars represent the standard deviation (SD).



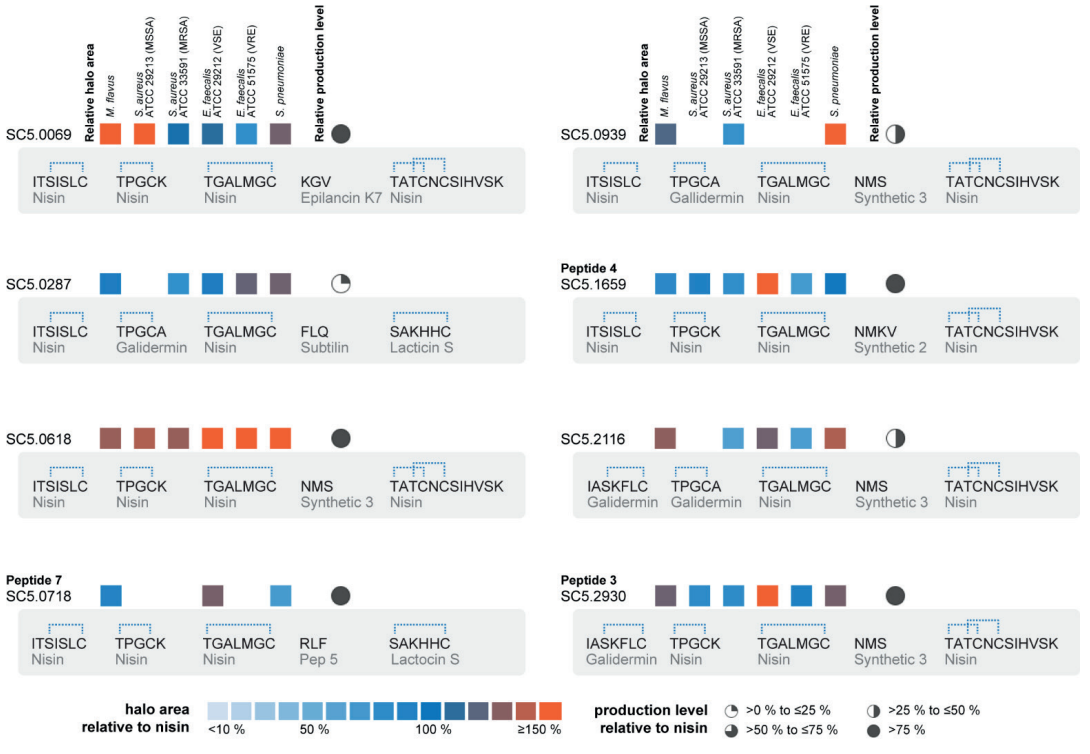
Supplementary Figure 5. Characterization of the nanoFleming assay. (a) A mixture of two different *L. lactis* strains was encapsulated. The first strain, *L. lactis* NZ9000 [pIL3BTC, pNZE3-*nisA-mcherry*] secretes prenisin (left side, '+'), and the second strain *L. lactis* NZ9000 [pIL3BTC, pNZE3-*rdm-mcherry*] does not secrete any antimicrobial peptide (right side, '-'). The cells were co-encapsulated with the NisP-producing sensor strain *L. lactis* NZ9000 [pNG-*nisTPtdgfp*], grown for 18 h in an hydrophobic phase, and then analyzed by large-particle flow cytometry, gated on the presence of a colony (as indicated by the red fluorescence of the colonies) and plotted for their green fluorescence levels. (b) Mean green fluorescent levels of the nLRs indicate a difference of approx. 25-fold between nLRs harboring a prenisin producing strain and nLRs that do not. Bars represent the mean of 71 (+) and 984 (-) nLRs (n = 71, n = 984), error bars represent the standard deviation (SD).



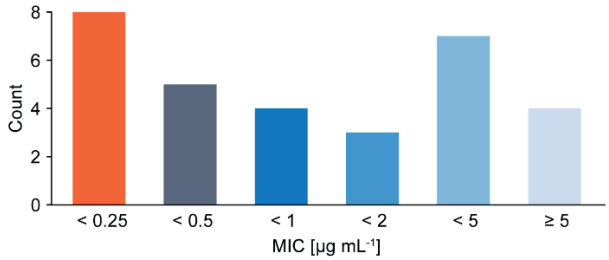
Supplementary Figure 6. Sensitivity of the nanoFleming assay. Three different nisin hinge region variants, NMK (nisin wild-type), GLV (nisin hinge-variant 1) and GGC (nisin hinge-variant 2) were produced by strain *L. lactis* NZ9000 [pIL3BTC, pNZE3-*nisA_x-mcherry*] (where x stands for the hinge region variant) and tested for their inhibition profiles against the NisP producing sensor strain *L. lactis* NZ9000 [pNG*nisTPtdgfp*] with two assay types: a standard inhibition zone assay performed on a petri dish and the nanoFleming assay. In the petri dish assay, the nisin wild-type resulted in the formation of a large halo whereas the two variants with modulated hinge regions showed a reduction in halo size. In fact, the hinge-variant 2 resulted in a halo just above the detection limit of the assay (orange arrows). When embedded in nLRs, results followed the same trend. The wild-type hinge region led to the strongest reduction in sensor growth (as measured by large-particle flow cytometry analyzing the green fluorescence of the sensor strain) here the mean green fluorescence intensity of the peak containing the nLRs with low sensor content (left fringe of the histogram) was the lowest for the wild-type hinge region (20 AU), while growth-reduction obtained from the hinge-variant 2 was considerably weaker (33 AU). However, the population not secreting a peptide (strain *L. lactis* NZ9000 [pIL3BTC, pNZE3-*rdm-mcherry*]) was well separated from the peptide secreting population in each of the three cases (>3 σ from the mean of the negative population), suggesting that the assay would also detect small amounts of antimicrobial activity.



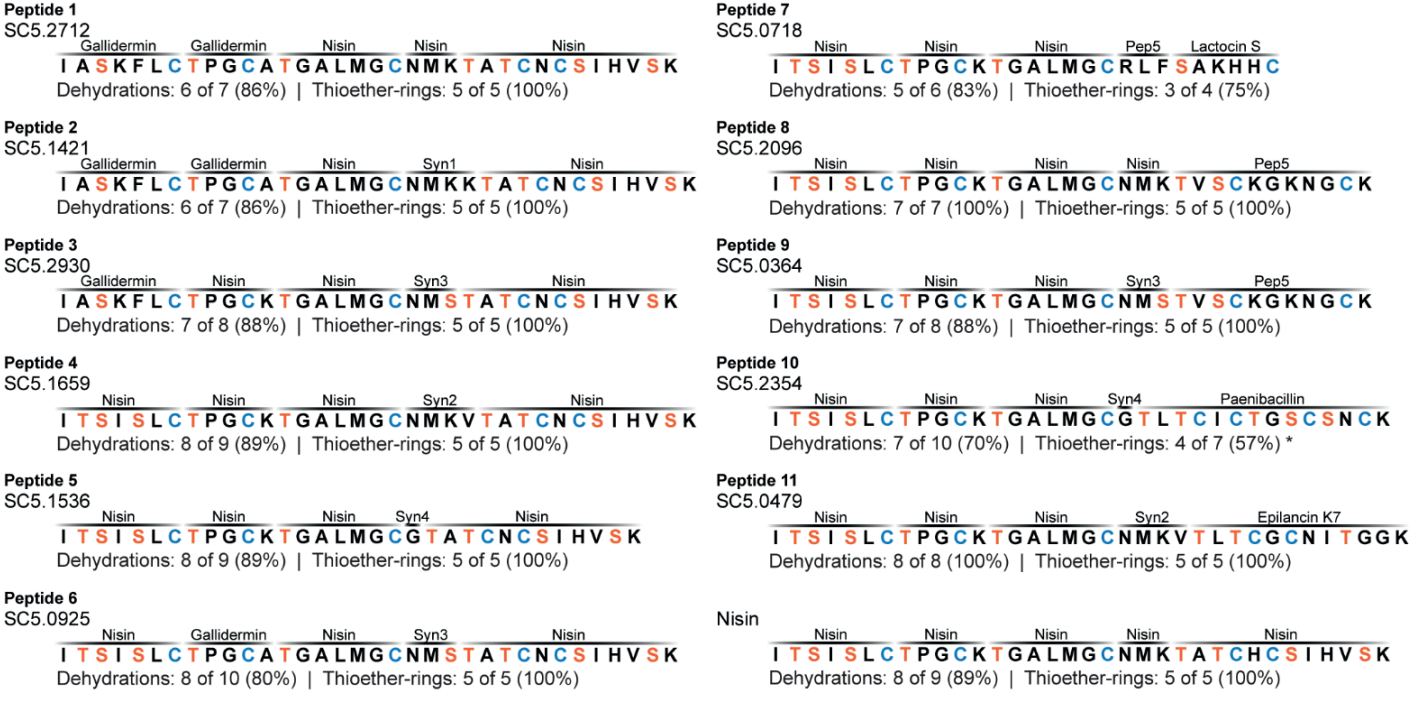
Supplementary Figure 7. Activity of peptide candidates against *M. flavus*. Summary of 126 antimicrobial peptides that were precipitated and their antimicrobial activities against *M. flavus* were determined by the size of the halo in an inhibition zone assay (100% = nisin). The production levels were approximated using HPLC-MSMS (100% = nisin).



Supplementary Figure 8. Activity of peptide candidates against reference strains. Summary of 8 antimicrobial peptides that were precipitated and their antimicrobial activities against different Gram-positive reference strains were determined by the size of the halo in an inhibition zone assay (100% = nisin). The production levels were approximated using HPLC-MSMS (100% = nisin). We observed peptides with larger halos for all tested strains, e.g. SC5.0618. Other peptides show clear preference for the inhibition of a certain stain, e.g. SC5.0939 that was specifically active against *S. pneumoniae* or SC5.0287 that has nisin-like activity against most strains but not against MSSA. All peptides were precipitated in duplicate (n = 2) and antimicrobial activity and production levels were quantified in triplicate. Each dot represents the mean of those measurements.

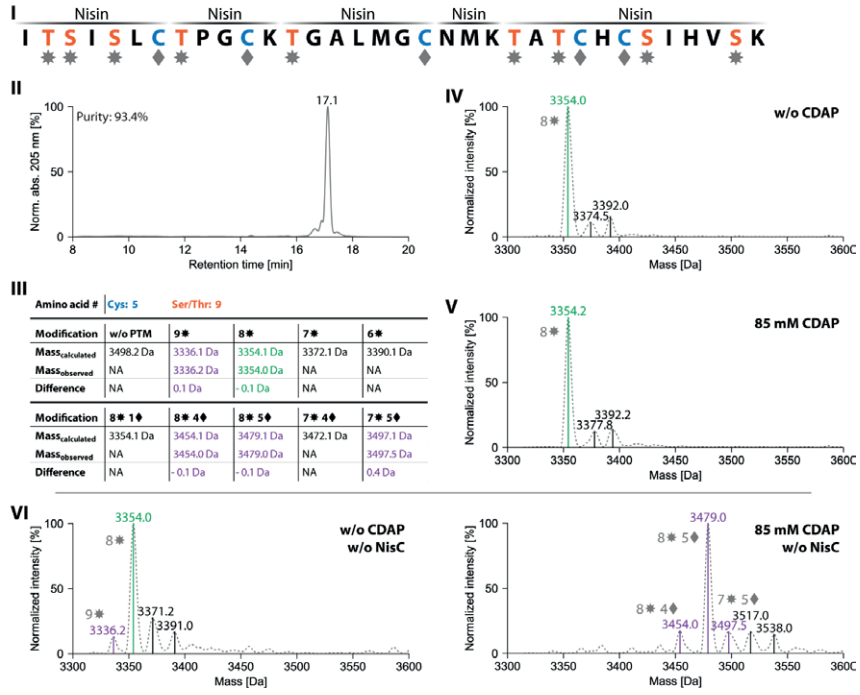


Supplementary Figure 9. MIC distribution of purified peptides against *M. flavus*. Distribution of minimal inhibitory concentrations of 31 purified peptides against *M. flavus*. Values in the histogram are means of three MIC experiments (n = 3).

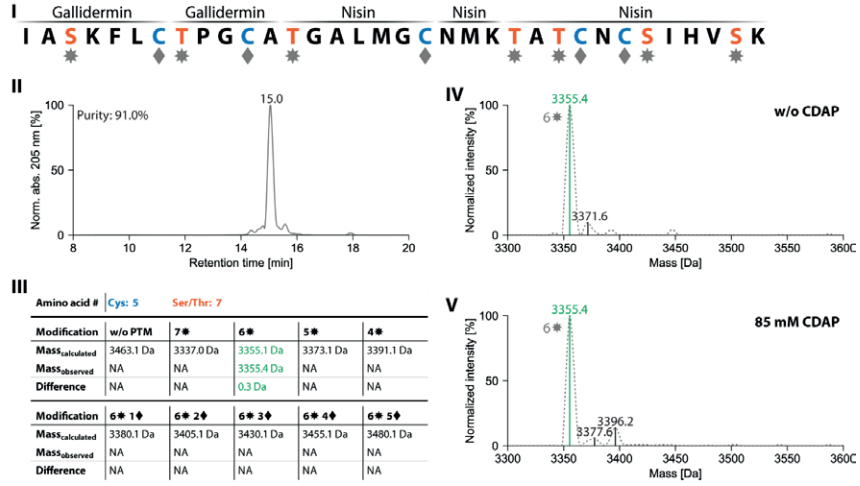


Supplementary Figure 10. Predominant modification pattern of selected peptides. Indicated are serines or threonines within the peptide that are putatively dehydrated (in orange) and can undergo thioether ring formation with cysteines (in blue). For every peptide the total number of dehydrations (each resulting in mass shift of -18 Da) was elucidated by mass spectrometry of the peptides. The number of thioether rings was detected indirectly by chemical derivatization of the peptides with 1cyano4dimethylaminopyridinium tetrafluoroborate (CDAP). CDAP only reacts with the (reduced) sulfhydryl group of cysteines, a mass shift of +25 Da therefore indicates a sulfhydryl group that is not involved in a thioether ring. In cases where variants contained a mixture of different modifications only the most predominant modification is listed. (*) Note that peptide 10 (SC5.2354) is a mixture of different dehydration and thioether patterns with a partially equal distribution (see also Supplementary Fig. 11).

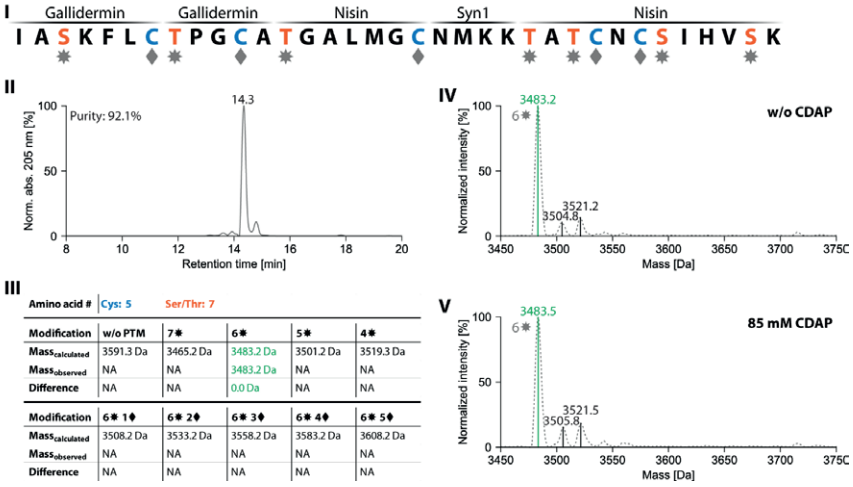
a Nisin



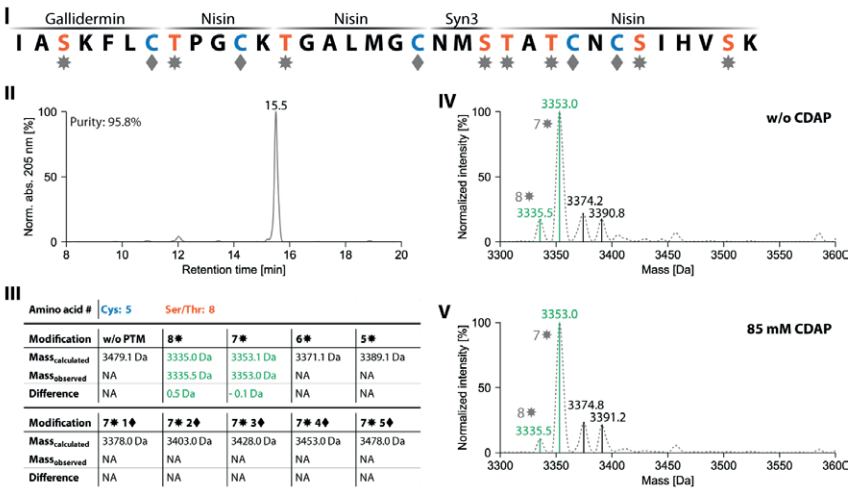
b **Peptide 1** SC5.2712



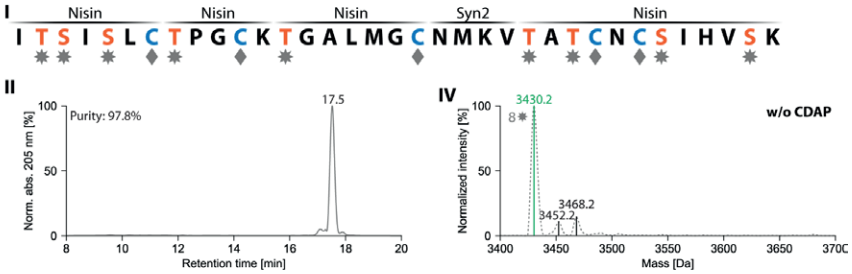
c **Peptide 2** SC5.1421



d **Peptide 3** SC5.2930



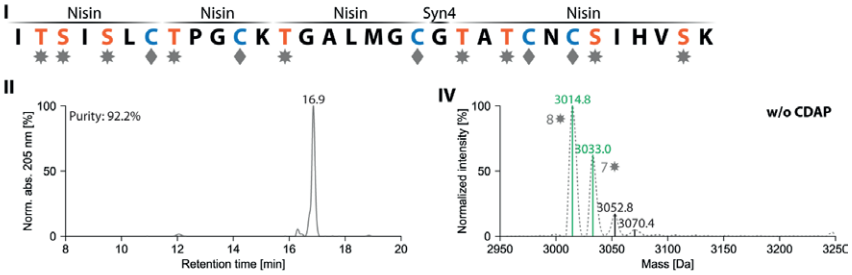
e **Peptide 4** SC5.1659



III

Amino acid #	Cys: 5	Ser/Thr: 9			
Modification	**w/o PTM**	**9✱**	**8✱**	**7✱**	**6✱**
Mass$_{calculated}$	3574.3 Da	3412.2 Da	3430.2 Da	3448.2 Da	3466.2 Da
Mass$_{observed}$	NA	NA	3430.2 Da	NA	NA
Difference	NA	NA	0.0 Da	NA	NA
Modification	**8✱ 1♦**	**8✱ 2♦**	**8✱ 3♦**	**8✱ 4♦**	**8✱ 5♦**
Mass$_{calculated}$	3455.2 Da	3480.2 Da	3505.2 Da	3530.2 Da	3555.2 Da
Mass$_{observed}$	NA	NA	NA	NA	NA
Difference	NA	NA	NA	NA	NA

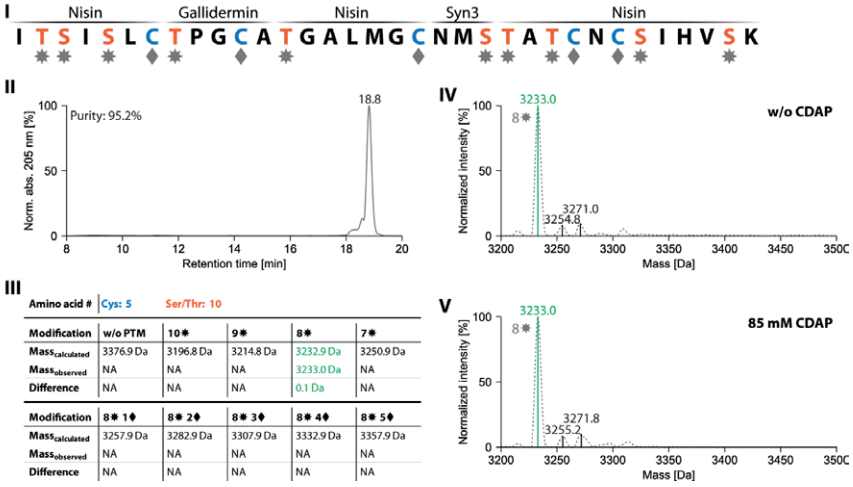
f **Peptide 5** SC5.1536



III

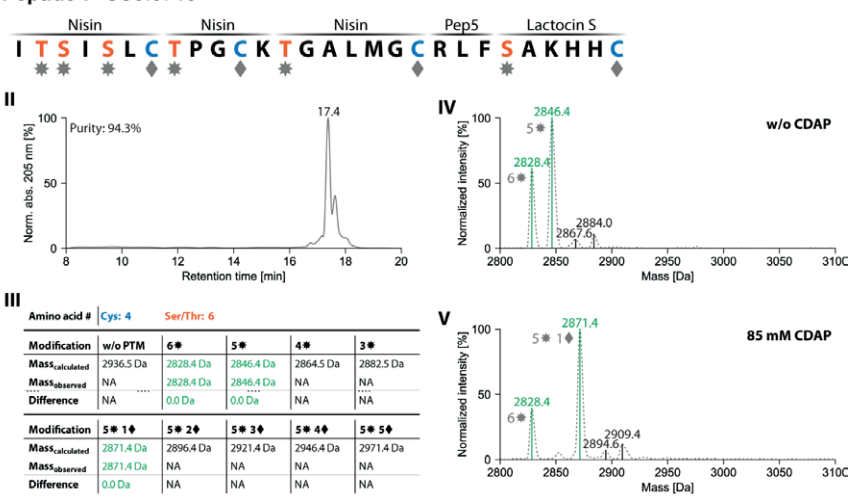
Amino acid #	Cys: 5	Ser/Thr: 9			
Modification	**w/o PTM**	**9✱**	**8✱**	**7✱**	**6✱**
Mass$_{calculated}$	3158.7 Da	2996.6 Da	3014.6 Da	3032.6 Da	3050.7 Da
Mass$_{observed}$	NA	NA	3014.8 Da	3033.0 Da	NA
Difference	NA	NA	0.2 Da	0.4 Da	NA
Modification	**8✱ 1♦**	**8✱ 2♦**	**8✱ 3♦**	**7✱ 1♦**	**7✱ 2♦**
Mass$_{calculated}$	3039.6 Da	3064.6 Da	3089.6 Da	3057.6 Da	3082.6 Da
Mass$_{observed}$	NA	NA	NA	3057.8 Da	NA
Difference	NA	NA	NA	0.2 Da	NA

g **Peptide 6** SC5.0925



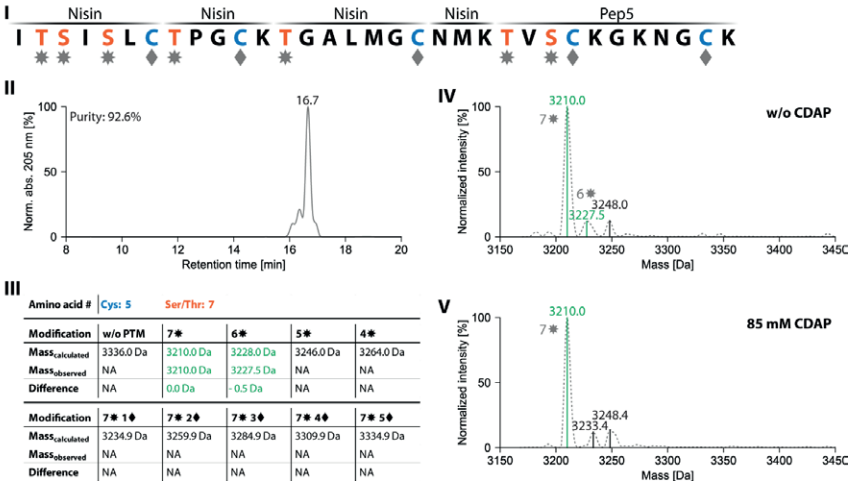
Amino acid #	Cys: 5	Ser/Thr: 10			
Modification	w/o PTM	10✱	9✱	8✱	7✱
$Mass_{calculated}$	3376.9 Da	3196.8 Da	3214.8 Da	3232.9 Da	3250.9 Da
$Mass_{observed}$	NA	NA	NA	3233.0 Da	NA
Difference	NA	NA	NA	0.1 Da	NA
Modification	8✱ 1♦	8✱ 2♦	8✱ 3♦	8✱ 4♦	8✱ 5♦
$Mass_{calculated}$	3257.9 Da	3282.9 Da	3307.9 Da	3332.9 Da	3357.9 Da
$Mass_{observed}$	NA	NA	NA	NA	NA
Difference	NA	NA	NA	NA	NA

h **Peptide 7** SC5.0718



Amino acid #	Cys: 4	Ser/Thr: 6			
Modification	w/o PTM	6✱	5✱	4✱	3✱
$Mass_{calculated}$	2936.5 Da	2828.4 Da	2846.4 Da	2864.5 Da	2882.5 Da
$Mass_{observed}$	NA	2828.4 Da	2846.4 Da	NA	NA
Difference	NA	0.0 Da	0.0 Da	NA	NA
Modification	5✱ 1♦	5✱ 2♦	5✱ 3♦	5✱ 4♦	5✱ 5♦
$Mass_{calculated}$	2871.4 Da	2896.4 Da	2921.4 Da	2946.4 Da	2971.4 Da
$Mass_{observed}$	2871.4 Da	NA	NA	NA	NA
Difference	0.0 Da	NA	NA	NA	NA

I Peptide 8 SC5.2096

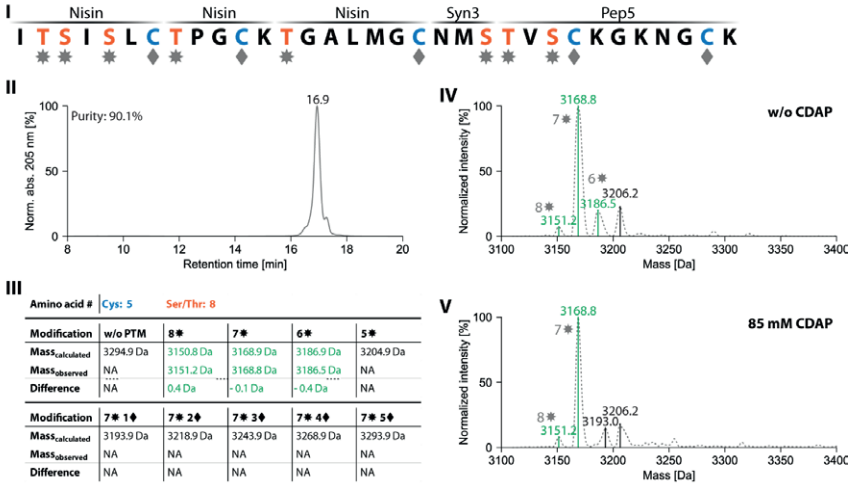


III

Amino acid #	Cys: 5	Ser/Thr: 7			
Modification	**w/o PTM**	**7✱**	**6✱**	**5✱**	**4✱**
Mass$_{calculated}$	3336.0 Da	3210.0 Da	3228.0 Da	3246.0 Da	3264.0 Da
Mass$_{observed}$	NA	3210.0 Da	3227.5 Da	NA	NA
Difference	NA	0.0 Da	- 0.5 Da	NA	NA

Modification	**7✱ 1♦**	**7✱ 2♦**	**7✱ 3♦**	**7✱ 4♦**	**7✱ 5♦**
Mass$_{calculated}$	3234.9 Da	3259.9 Da	3284.9 Da	3309.9 Da	3334.9 Da
Mass$_{observed}$	NA	NA	NA	NA	NA
Difference	NA	NA	NA	NA	NA

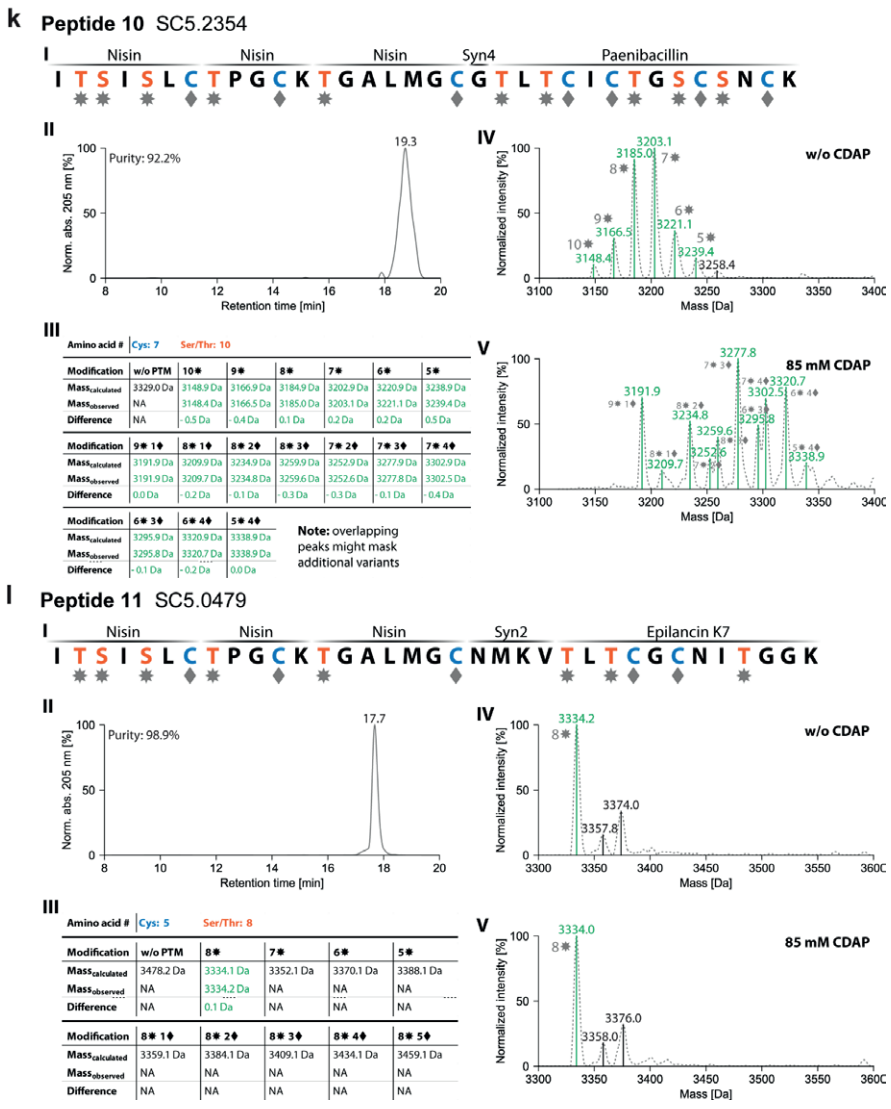
j Peptide 9 SC5.0364



III

Amino acid #	Cys: 5	Ser/Thr: 8			
Modification	**w/o PTM**	**8✱**	**7✱**	**6✱**	**5✱**
Mass$_{calculated}$	3294.9 Da	3150.8 Da	3168.9 Da	3186.9 Da	3204.9 Da
Mass$_{observed}$	NA	3151.2 Da	3168.8 Da	3186.5 Da	NA
Difference	NA	0.4 Da	- 0.1 Da	- 0.4 Da	NA

Modification	**7✱ 1♦**	**7✱ 2♦**	**7✱ 3♦**	**7✱ 4♦**	**7✱ 5♦**
Mass$_{calculated}$	3193.9 Da	3218.9 Da	3243.9 Da	3268.9 Da	3293.9 Da
Mass$_{observed}$	NA	NA	NA	NA	NA
Difference	NA	NA	NA	NA	NA



Supplementary Figure 11. Mass spectrometric characterization of selected peptides. Masses and PTMs of 12 peptides I: Amino acid sequence of the peptide; serines and threonines (in orange) can be dehydrated by NisB resulting in a mass shift of −18 Da (✱) whereas cysteine residues (in blue) that are not involved in thioether ring formation catalyzed by NisC and thus carry sulfhydryl groups can be derivatized by CDAP resulting in a mass shift of +25 Da (♦). Cysteines that do not undergo derivatization by CDAP are used to calculate the number of thioether rings that must have been formed. II: Chromatogram at 205 nm of the HPLC purified peptide. Purity at 205 nm for the main peptide peak. III: Calculated peptide masses compared to the values observed by mass spectrometry. Matches of calculated vs. observed masses (difference ≤0.5 Da) are indicated in green IV: Mass spectrum of the peptide, peaks matching the calculated peptide mass are highlighted in green. V: Mass spectrum after treatment with CDAP, peaks matching the calculated peptide mass are highlighted in green. (a) Characterization of nisin. To prove derivatization of the sulfhydryl groups by CDAP, a nisin peptide produced by a strain lacking the NisC enzyme (*L. lactis* NZ9000 [pIL3BT]) was used (VI). (b-l) Characterization of the combinatorial peptides.

Supplementary Table 1. Wild-type peptide sequences used in this study.

Peptide	Sequence
Nisin	I T S I S L C T P G C K T G A L M G C N M K T A T C H C S I H V S K
Subtilin	W K S E S L C T P GC V T G A L Q T C F L Q T L T C N C K I S K
Gallidermin	I A S K F LC T P G C A K T G S F N S Y C C
Mutacin B-Ny266	F K S W S F C T P G CA K T G S F N S Y C C
Pep5	T A G P A I R A S V K Q C Q K T L K A T R L F T V S C K G K N G C K
Epicidin 280	S L G P A I K A T R Q V C P K A T R F V T VS C K K S D C Q
Epilancin K7	S A S V L K T S I KV S K K Y C K G V T L T C G C N I T G G K
Paenibacillin	A S I I K T T I K V S K A V C K T L T C I C T G S C S N C K
Lacticin 481	K G G S G V I H T I S H E C N M N S W Q F V F T C C S
Actagardine	S S G W V C T L T I E C G T V I C A C
Lactocin S	S T P V L A S V A V S M E L L P T A S V L Y S D V A G C F K Y S A K H H C
Haloduracin α	C A W Y N I S C R L G N K G A Y C T L T V E C M P S C N

Underlined amino acids indicate the sequences used for the definition of peptide modules.

Supplementary Table 2. MICs of combinatorial peptides against *M. flavus*.

	Peptide	Module combination B1	B2	P1	P2	P3	MIC [μg mL^{-1}] *M. flavus*
	SC5.0069	Nisin	Nisin	Nisin	Epilancin K7	Nisin	0.27
	SC5.0086	Gallidermin	Gallidermin	Nisin	Synthetic 3	Nisin	0.59
	SC5.0189	Nisin	Nisin	Nisin	Synthetic 3	Epilancin K7	3.37
	SC5.0333	Nisin	Nisin	Nisin	Pep 5	Other	0.49
9	SC5.0364	Nisin	Nisin	Nisin	Synthetic 3	Pep 5	0.44
11	SC5.0479	Nisin	Nisin	Nisin	Synthetic 2	Epilancin K7	1.03
	SC5.0487	Nisin	Gallidermin	Actagardine	Epilancin K7	Pep 5	3.82
	SC5.0515	Nisin	Gallidermin	Nisin	Gallidermin	Lactocin S	3.03
	SC5.0531	Gallidermin	Gallidermin	Mutacin B-Ny266	Synthetic 4	Epilancin K7	0.55
7	SC5.0718	Nisin	Nisin	Nisin	Pep 5	Lactocin S	0.45
6	SC5.0925	Nisin	Gallidermin	Nisin	Synthetic 3	Nisin	0.87
	SC5.0953	Gallidermin	Nisin	Nisin	Synthetic 2	Nisin	0.08
	SC5.1048	Gallidermin	Nisin	Actagardine	Synthetic 3	Epilancin K7	14.1
	SC5.1237	Nisin	Nisin	Actagardine	Synthetic 3	Nisin	2.08
2	SC5.1421	Gallidermin	Gallidermin	Nisin	Synthetic 1	Nisin	0.21
	SC5.1491	Nisin	Gallidermin	Nisin	Epilancin K7	Nisin	5.11
	SC5.1516	Nisin	Gallidermin	Actagardine	EpilancinK7	Lactocin S	2.90
5	SC5.1536	Nisin	Nisin	Nisin	Synthetic 4	Nisin	0.59
	SC5.1620	Nisin	Gallidermin	Nisin	Synthetic 1	Nisin	0.33
4	SC5.1659	Nisin	Nisin	Nisin	Synthetic 2	Nisin	0.14
	SC5.1743	Nisin	Nisin	Nisin	Synthetic 3	Nisin	0.14
	SC5.1922	Gallidermin	Gallidermin	Actagardine	Synthetic 3	Epilancin K7	33.8
8	SC5.2096	Nisin	Nisin	Nisin	Nisin	Pep 5	0.18
10	SC5.2354	Nisin	Nisin	Nisin	Synthetic 4	Paenibacillin	2.95
	SC5.2630	Nisin	Nisin	Nisin	Synthetic 4	Epilancin K7	2.29
	SC5.2701	Nisin	Nisin	Paenibacillin	Synthetic 1	Lactocin S	1.49
1	SC5.2712	Gallidermin	Gallidermin	Nisin	Nisin	Nisin	0.13
	SC5.2829	Nisin	Gallidermin	Nisin	Nisin	Nisin	0.15
3	SC5.2930	Gallidermin	Nisin	Nisin	Synthetic 3	Nisin	0.04
	SC5.9487	Gallidermin	Epicidin 280	Paenibacillin	Pep 5	Nisin	1.14
	SC5.9925	Nisin	Haloduracin α	Lactocin S	Synthetic 4	Epilancin K7	73.6
	Nisin						0.07

Supplementary Table 3. DNA oligonucleotides used in this study.

ID	Name	Sequence (5' → 3')
1	*lib-2nd-fw*	GGTGCTAGCCCACGTATT
2	*ccdb-BamHI-NheI-fw*	GGATCCTCTAGAGCTAGCCCGGAATTGCCAGCTGGGGCGC
3	*ccdb-HindIII-rv*	GGCCGCAAGCTTTTATTAAATGCCCCAAAACA
4	*pSEVA-t1-fw*	TACTCAGGAGAGCGTTCACC
5	*pSEVA-to-rv*	GGGGACCCCTGGATTCTCAC
6	*mcherry-NdeI-fw*	TGCAGCTcatatgCATAGCAAGGGC
7	*mcherry-HindIII-BamHI-rv*	TGAGCAaagcttggatccTTATTACTTGTACAGCTC
8	*P23-HindIII-NheI-fw*	TAATATaagcttgctagcTCGAAAAGCCCTG
9	*P23-NdeI-rv*	AGCGCAcatatgATCATTGTCATTCATATTTT
10	*pNZE3pNG-NheI-fw*	TAATGGTCTCTgtatacGCTAGCttctcgagtgcatattttc
11	*pNZE3pNG-BamHI-rv*	TAATGGTCTCTgtatacGGATCCgttatcggtcctttaattg
12	*mcherry-NheI-rm-fw*	GTCAGGGCTTTTCGAGGCAGCttctcgagtgc
13	*mcherry-NheI-rm-rv*	gcactcgagaaGCTGCCTCGAAAAGCCCTGAC
14	*nis-fragment-BglII-HindIII*	agatctagtcttataactatactgacaatagaaacattaacaaatctaaaacagtcttaattctatct tgagaaagtattggtaataatattattgtcgataacgcgagcataataaacggctctgattaaattc tgaagtttgttagatacaatgatttcgttcgaaggaactacaaaataaattataaggaggcactca ccatgtcaacaaaagattttaatcttgatcttgtttcagtttcaaaaaaagattcaggtgctagccca cgtattacttcaatttcactttgtactccaggttgtaaaactggtgctcttatgggttgtaatatgaa aactgctacttgtcattgttcaattcatgtttcaaaatgataagctt
15	*rdm-NheI-HindIII-fw*	[P]ctagcTAATCATCTAACATGGGATTTGCTATAACTCTTGAA CGCTACATGTACGAAACCATATTAa
16	*rdm-NheI-HindIII-rv*	[P]agcttTAATATGGTTTCGTACATGTAGCGTTCAAGAGTTAT AGCAAATCCCATGTTAGATGATTAg
17	*pNZE3-seq-fw*	aaacatgcaggaattgacga
18	*pNZE3-seq-rv*	ggcttttacgctacgataac
19	*tag-HisW-BsaI-fw*	atcttcGGTCTCtCATCACCATCACTGGtcaacaaaagattttaatcttg
20	*tag-HisW-BsaI-rv*	tccaatGGTCTCtGATGGTGATGcatggtgagtgcctcc
21	*tdgfp-NdeI-fw*	TGCAGCTcatatgAGTAAACGAGAAGAACT
22	*tdgfp-HindIII-BamHI-rv*	TGAGCAaagcttggatccTTATTTGTAGAGCTCAT

Underlined bases indicate restriction sites (see oligonucleotide name for enzyme).
[P] indicates a phosphorylation of the hydroxyl group at the terminal base of the oligonucleotide.

Supplementary Table 4. Bacterial strains used in this study.

ID	Name	Genotype	Reference
1	*M. flavus* NIZO B423		NIZO food research
2	*L. lactis* NZ9000	MG1655 *pepN*::*nisRnisK*	1
3	*L. lactis* NZ9800	NZ9700 Δ*nisA*	2
4	*L. lactis* NZ9803	NZ9800 Δ*nisP* Δ*nisA*	This work
5	*S. aureus* ATCC 29213	methicillin sensitive (MSSA)	3
6	*S. aureus* ATCC 33591	methicillin resistant (MRSA)	3,4
7	*S. aureus* CAL	methicillin resistant (MRSA)	The University Medical Center Groningen, The Netherlands
8	*S. aureus* MW2	methicillin resistant (MRSA)	The University Medical Center Groningen, The Netherlands
9	*E. faecalis* ATCC 29212	vancomycin sensitive (VSE)	3
10	*E. faecalis* ATCC 51575	gentamicin, streptomycin, and vancomycin resistant (VRE)	3,5
11	*E. faecalis* LMG 16216	vancomycin resistant (VRE)	Laboratory of Microbiology, Gent, Belgium
12	*E. faecium* LMG 16003	avoparcin and vancomycin resistant (VRE)	Laboratory of Microbiology, Gent, Belgium
13	*S. pneumoniae* ATCC 49619	serotype 19F	3
14	*S. pneumoniae* D39	serotype 2	6
15	*S. pneumoniae* TIGR4	serotype 4	7
16	*B. cereus* ATCC 14579		8
17	*E. coli* DH5α	F^- Φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(rK^-, mK^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	9
18	*E. coli* DB3.1	F^- *gyrA462 endA1 glnV44* Δ(*sr1-recA*) *mcrB mrr hsdS20*(rB^-, mB^-) *ara14 galK2 lacY1 proA2 rpsL20*(Sm^r) *xyl5* Δ*leu mtl1*	10,11

Supplementary Table 5. Plasmids used in this study.

ID	Name	Features	Reference
1	pIL3BTC	pAMβ1 origin of replication, chloramphenicol resistance, expression of *nisBTC* under control of the P*nis* promoter	12
2	pNSR	pNZ-SV-*Sa*NSR, pSH71 origin of replication, chloramphenicol resistance, *L. lactis – E. coli* shuttle, expression of *nsr* under control of the P*nis* promoter	13,14
3	pEmpty	pNZ8048, pSH71 origin of replication, chloramphenicol resistance, *L. lactis – E. coli* shuttle, P*nis* promoter and empty multiple cloning site	1
4	pQL11	pUC origin of replication, ampicillin resistance, carries the *ccdB* expression cassette	15
5	pSEVA241	ColE1 origin of replication, kanamycin resistance	16
6	pSEVA241-*silent*	as pSEVA241, carries cassette for *ccdB* expression	This work
7	pSEVA241-*library*	as pSEVA241, carries the leader-less library	This work
8	pNZE3-*nisA*	pSH71 origin of replication, erythromycin resistance, *L. lactis – E. coli* shuttle, expression of *nisA* under control of the P*nis* promoter	17
9	pSB1C3-BBa_J06504	pMB1 origin of replication, chloramphenicol resistance, carries the gene for mCherry (BioBrick part BBa_J06504)	18,19
10	pNZE3-*nisA-mcherry*	as pNZE3-*nisA*, expression of *mcherry* under control of the P23 promoter	This work
11	pMA-T-*nisAopt*	pRO1600/ColE1 origin of replication, ampicillin resistance, carries a codon-optimized nisin leader and *nisA* gene	This work
12	pNZE3-*nisA-opt-mcherry*	as pNZE3-*nisA-mcherry*, carries a codon-optimized nisin leader and *nisA* gene separated by a NheI site for cloning and fused to the P*nis* promoter	This work
13	pNZE3-*rdm-mcherry*	as pNZE3-*nisAopt-mcherry*, carries a random sequence instead of the *nisA* structural gene	This work
14	pNZE3-*library-mcherry*	as pNZE3-*nisAopt-mcherry*, carries the library instead of the *nisA* structural gene	This work
15	pIL3BT	pIL3BTC derived, lacking the gene for NisC	20
16	pNZ-*nisPsol*-8H	pSH71 origin of replication, chloramphenicol resistance, *L. lactis – E. coli* shuttle, expression of *nisP* with His_8-tag under control of the P*nis* promoter	21
17	pNG-*nisTP*	pSH71 origin of replication, chloramphenicol resistance, *L. lactis – E. coli* shuttle, expression of *nisP* under control of the P*nis* promoter	17
18	pKQV5-*tdgfp*	pBR322 origin of replication, ampicillin resistance, expression of *tdgfp* under control of the Ptac promoter	22
19	pNG-*nisTP-tdgfp*	as pNG-*nisTP*, expression of *tdgfp* under control of the P23 promoter	This work

Supplementary Table 6. DNA sequences of peptide modules.

Origin	5'-const.	Module B1	Module B2	Module P1	Module P2	Module P3	3'-const.
Constant regions	GGTGCTAGC CCACGT						TGATAAGCTT TCTTTGAAC
Nisin		ATTACTTCGAT CTCATTGTGT	ACACCTGGTT GTAAA	ACAGGTGCACT TATGGGTTGT	AATATGAAA	ACAGCTACTTGTAATTGT TCAATTCACGTTTCAAAA	
Subtilin				TGGAAATCAGA ATCTCTTTGC	TTCCTTCAA		
Gallidermin		ATTGCATCAAA ATTTCTTTGT	ACTCCAGGTT GTGCA		AAAACAGGT		
Mutacin B-Ny266				TTTAAATCTTGG AGCTTTTGT			
Pep5			TCAGTAAAAC AATGTCAA	AAAACATTAAAA GCTACA	CGCCTTTTT	ACAGTTTCATGTAAAGGT AAAAACGGATGTAAA	
Epicidin 280			ACACGTCAAG TTTGTCCA				
Epilancin K7				TCAAAAAAATAT TGC	AAAGGTGTT	ACATTGACATGTGGATGC AACATCACAGGAGGTAAA	
Paenibacillin			ACTACAATTA AAGTT	TCAAAAGCAGTT TGT		ACATTGACATGTATTTGTACA GGTTCATGTTCAAATTGTAAG	
Lacticin 481				AAAGGTGGTTCT GGAGTTATTCAT			
Actagardine				TCATCAGGTTGG GTTTGC			
Lactocin S				TCTGATGTGGCTG GCTGT	TTCAAATAT	TCAGCTAAACATCACTGC	
Halodura-cin α			ACTTTGACAG TTGAGTGT				
Empty				n/a			
Synthetic 1					AATATGAAAAAA		
Synthetic 2					AACATGAAAGTC		
Synthetic 3					AATATGTCA		
Synthetic 4					GGT		

Underlined bases indicate restriction sites; "n/a" indicates an empty module position that is left out during synthesis.

Supplementary Table 7. Amino acid and DNA sequences of combinatorial peptides.

ID	Sequence
SC5.0069	**AA:** ITSISLCTPGCKTGALMGCKGVTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAAAGGTGTTACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.0086	**AA:** IASKFLCTPGCATGALMGCNMSTATCNCSIHVSK
	DNA: ATTGCATCAAAATTTCTTTGTACTCCAGGTTGTGCAACAGGTGCACTTATG GGTTGTAATATGTCAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.0189	**AA:** ITSISLCTPGCKTGALMGCNMSTLTCGCNITGGK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAATATGTCAACATTGACATGTGGATGCAACATCACAGGAGGTAAA
SC5.0333	**AA:** ITSISLCTPGCKTGALMGCRLFTVSCKGKKRM
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTCGCCTTTTTACAGTTTCATGTAAAGGTAAAAAACGGATG
Peptide 9 SC5.0364	**AA:** ITSISLCTPGCKTGALMGCNMSTVSCKGKNGCK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAATATGTCAACAGTTTCATGTAAAGGTAAAAACGGATGTAAA
Peptide 11 SC5.0479	**AA:** ITSISLCTPGCKTGALMGCNMKVTLTCGCNITGGK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAACATGAAAGTCACATTGACATGTGGATGCAACATCACAGGAGGTAAA
SC5.0487	**AA:** ITSISLCTPGCASSGWVCKGVTVSCKGKNGCK
	DNA: ATTACTTCGATCTCATTGTGTACTCCAGGTTGTGCATCATCAGGTTGGGTT TGCAAAGGTGTTACAGTTTCATGTAAAGGTAAAAACGGATGTAAA
SC5.0515	**AA:** ITSISLCTPGCATGALMGCKTGSAKHHC
	DNA: ATTACTTCGATCTCATTGTGTACTCCAGGTTGTGCAACAGGTGCACTTATG GGTTGTAAAACAGGTTCAGCTAAACATCACTGC
SC5.0531	**AA:** IASKFLCTPGCAFKSWSFCGTLTCGCNITGGK
	DNA: ATTGCATCAAAATTTCTTTGTACTCCAGGTTGTGCATTTAAATCTTGGAGC TTTTGTGGTACATTGACATGTGGATGCAACATCACAGGAGGTAAA
Peptide 7 SC5.0718	**AA:** ITSISLCTPGCKTGALMGCRLFSAKHHC
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTCGCCTTTTTTCAGCTAAACATCACTGC
Peptide 6 SC5.0925	**AA:** ITSISLCTPGCATGALMGCNMSTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACTCCAGGTTGTGCAACAGGTGCACTTATG GGTTGTAATATGTCAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.0953	**AA:** IASKFLCTPGCKTGALMGCNMKVTATCNCSIHVSK
	DNA: ATTGCATCAAAATTTCTTTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAACATGAAAGTCACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.1048	**AA:** IASKFLCTPGCKSSGWVCNMSTLTCGCNITGGK
	DNA: ATTGCATCAAAATTTCTTTGTACACCTGGTTGTAAATCATCAGGTTGGGTT TGCAATATGTCAACATTGACATGTGGATGCAACATCACAGGAGGTAAA
SC5.1237	**AA:** ITSISLCTPGCKSSGWVCNMSTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAATCATCAGGTTGGGTT TGCAATATGTCAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
Peptide 2 SC5.1421	**AA:** IASKFLCTPGCATGALMGCNMKKTATCNCSIHVSK
	DNA: ATTGCATCAAAATTTCTTTGTACTCCAGGTTGTGCAACAGGTGCACTTATG GGTTGTAATATGAAAAAAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA

ID	Sequence
SC5.1491	**AA:** ITSISLCTPGCATGALMGCKGVTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACTCCAGGTTGTGCAACAGGTGCACTTATG GGTTGTAAAGGTGTTACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.1516	**AA:** ITSISLCTPGCASSGWVCKGVSAKHHC
	DNA: ATTACTTCGATCTCATTGTGTACTCCAGGTTGTGCATCATCAGGTTGGGTT TGCAAAGGTGTTTCAGCTAAACATCACTGC
Peptide 5 SC5.1536	**AA:** ITSISLCTPGCKTGALMGCGTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTGGTACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.1620	**AA:** ITSISLCTPGCATGALMGCNMKKTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACTCCAGGTTGTGCAACAGGTGCACTTATG GGTTGTAATATGAAAAAAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
Peptide 4 SC5.1659	**AA:** ITSISLCTPGCKTGALMGCNMKVTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAACATGAAAGTCACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.1743	**AA:** ITSISLCTPGCKTGALMGCNMSTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAATATGTCAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.1922	**AA:** IASKFLCTPGCASSGWVCNMSTLTCGCNITGGK
	DNA: ATTGCATCAAAATTTCTTTGTACTCCAGGTTGTGCATCATCAGGTTGGGTT TGCAATATGTCAACATTGACATGTGGATGCAACATCACAGGAGGTAAA
Peptide 8 SC5.2096	**AA:** ITSISLCTPGCKTGALMGCNMKTVSCKGKNGCK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAATATGAAAACAGTTTCATGTAAAGGTAAAAACGGATGTAAA
Peptide 10 SC5.2354	**AA:** ITSISLCTPGCKTGALMGCGTLTCICTGSCSNCK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTGGTACATTGACATGTATTTGTACAGGTTCATGTTCAAATTGTAAG
SC5.2630	**AA:** ITSISLCTPGCKTGALMGCGTLTCGCNITGGK
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTGGTACATTGACATGTGGATGCAACATCACAGGAGGTAAA
SC5.2701	**AA:** ITSISLCTPGCKSKAVCNMKKSAKHHC
	DNA: ATTACTTCGATCTCATTGTGTACACCTGGTTGTAAATCAAAAGCAGTTTGT AATATGAAAAAATCAGCTAAACATCACTGC
Peptide 1 SC5.2712	**AA:** IASKFLCTPGCATGALMGCNMKTATCNCSIHVSK
	DNA: ATTGCATCAAAATTTCTTTGTACTCCAGGTTGTGCAACAGGTGCACTTATG GGTTGTAATATGAAAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.2829	**AA:** ITSISLCTPGCATGALMGCNMKTATCNCSIHVSK
	DNA: ATTACTTCGATCTCATTGTGTACTCCAGGTTGTGCAACAGGTGCACTTATG GGTTGTAATATGAAAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
Peptide 3 SC5.2930	**AA:** IASKFLCTPGCKTGALMGCNMSTATCNCSIHVSK
	DNA: ATTGCATCAAAATTTCTTTGTACACCTGGTTGTAAAACAGGTGCACTTATG GGTTGTAATATGTCAACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.9487	**AA:** IASKFLCTRQVCPSKAVCRLFTATCNCSIHVSK
	DNA: ATTGCATCAAAATTTCTTTGTACACGTCAAGTTTGTCCATCAAAAGCAGTT TGTCGCCTTTTTACAGCTACTTGTAATTGTTCAATTCACGTTTCAAAA
SC5.9925	**AA:** ITSISLCTLTVECSDVAGCGTLTCGCNITGGK
	DNA: ATTACTTCGATCTCATTGTGTACTTTGACAGTTGAGTGTTCTGATGTGGCT GGCTGTGGTACATTGACATGTGGATGCAACATCACAGGAGGTAAA

Supplementary References

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CHAPTER 4

Generation of nisin derivatives with an altered spectrum by incorporating methionine analogues

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Submitted

Abstract

Incorporation of non-canonical amino acids (ncAAs) into ribosomally synthesized and post-translationally modified peptides (RiPPs) is a promising strategy to produce RiPP derivatives with enhanced biological activity and altered physicochemical properties. The co-translational insertion of ncAAs together with post-translational modifications could dramatically expand the chemical and functional space of RiPPs, allowing for generating novel RiPP derivatives with improved specificity, stability and activity. Lantibiotics are an important class of RiPPs, which exhibit potent antimicrobial activity against some clinically relevant Gram-positive pathogens. Here, four methionine analogues with unsaturated and varying side chain length were successfully incorporated at four different positions of lantibiotic nisin in *Lactococcus lactis* through force feeding. This approach allows for residue-specific incorporation of methionine analogues into nisin to expand their structural diversity, alter their activity against pathogenic bacteria and alter their activity profiles. In addition, the insertion of methionine analogues with biorthogonal chemical reactivity, e.g. azidohomoalanine and homopropargylglycine, into nisin provides the opportunity for chemical coupling at various positions using a variety of ligands such as peptide moieties, antimicrobial moieties and/or fluorophores.

Keywords: lantibiotic, nisin, methionine analogues, *in vivo* incorporation, non-canonical amino acids, antimicrobial activity, incorporation efficiency

Introduction

Antimicrobial resistance is an increasingly serious global public health threat, as a growing number of infectious diseases are becoming more difficult to treat.[1] Great efforts have been made to look for alternative approaches using new molecules, especially those with new modes of action, to tackle antibiotic resistance.[2] Interest in peptide-based therapeutics has greatly increased in recent years.[3] Over 60 peptide drugs are currently approved by US Food and Drug Administration, and the number of peptides annually entering clinical trials is increasing.[4] The genome sequencing efforts of the last decades have uncovered a large group of peptides with broad structural diversity and potent biological activities, which are the ribosomally synthesized and post-translationally modified peptides (RiPPs). These peptides have been mainly found in bacteria, and to a lesser degree in fungi, animals and plants. The rapidly growing number of discovered RiPPs and their remarkable structural and functional diversity may lead to new pharmaceutical applications. Their unique biosynthetic pathways and relatively low genetic complexity of biosynthesis make them excellent candidates for synthetic biology and bioengineering.[5]

Several strategies have been applied to develop novel RiPP derivatives, such as site-directed mutagenesis[6], semi-synthesis[7-10], and total synthesis[11]. Incorporation of non-canonical amino acids (ncAAs) is a promising approach to generate novel peptide derivatives with enhanced bioactivity and physicochemical properties.[12-14] It is increasingly used in peptide and protein engineering.[15] The insertion of natural/synthetic ncAAs during translation could expand the scope of ribosomal peptide synthesis based on the 20 canonical amino acids.[16-19] More interestingly, the ncAAs with reactive groups (e.g. alkyne or azide) can be used as chemical handles for click chemistry. Up to date, over 150 ncAAs have been successfully incorporated into recombinant peptides and proteins.[20] Two *in vivo* approaches have been developed for incorporating ncAAs into peptides.[12,21] The first approach is "residue-specific incorporation". This method typically involves replacing natural amino acids with the ncAAs of interest by using auxotrophic strains. Only amino acid analogues that are structurally similar to the natural amino acids can be incorporated in this way. The other method to incorporate ncAAs in peptides is "site-specific incorporation". For this method, the co-expression of orthogonal amber suppressor aminoacyl-tRNA synthetase (AARS/tRNA) pairs is necessary. Specific mutations can be introduced into the peptide sequence by reassigning the amber non-sense stop codon during translation. However, the screening and development of orthogonal AARS/tRNA pairs is time-consuming and the production yield of this method is extremely low.[22] Therefore, the residue specific incorporation method is a more promising approach, as it is capable of generating broad structural diversity by directly incorporating ncAAs via translation into bioactive RiPPs.

Lantibiotics are a class of RiPPs, harbouring unusual amino acids such as lanthionine (Lan), methyllanthionine (MeLan), dehydroalanine (Dha) and dehydrobutyrine (Dhb).[23] Nisin, the first discovered and the best studied lantibiotic produced by *Lactococcus lactis*, has been used as a powerful and safe preservative against food spoilage bacteria for over 50 years.[24] Besides its preservative properties, nisin is effective against many antibiotic-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE). Nisin, encoded by *nisA* as a linear precursor peptide (57 aa) with a leader peptide (23 aa) attached, is released after modification and cleavage of the leader (34 aa). After ribosomal synthesis of the precursor peptide, the unmodified prenisin is processed by the modification machinery. Firstly, the serine and threonine residues in the core peptide are dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb) by the dehydratase NisB. The dehydrated residues are then specifically coupled to cysteine by the cyclase NisC to form lanthionine rings. Subsequently, the modified prenisin is transported out of the cell by the ABC-type transporter NisT, and then the leader is cleaved off by the extracellular protease NisP to liberate the active peptide.[24] Interestingly, this machinery has a broad substrate specificity, which allows for the divergence from the original core-peptide.

Several studies have shown that site-directed mutagenesis of nisin can enhance its bioactivity.[25] The mutation at sites I4, M17 and M21 of nisin could retain or even increase its antimicrobial activity against Gram-positive bacteria including *L. lactis* and *Listeria monocytogenes*.[25] Here, we describe the incorporation of six methionine analogues with unsaturated, unique chemical handles and varying side chain length, i.e. Aha (azidohomoalanine), Hpg (homopropargylglycine), Nle (norleucine), Eth (ethionine), Nva (norvaline), and Alg (allyglycine), at four different positions of the lantibiotic nisin by using a methionine auxotrophic strain *Lactococcus lactis*. To test the effect of single methionine analogue replacement, four single Met nisin mutants, i.e. M17I, M21V, M17I-M21V-M35, and I4M-M17I-M21V, were constructed. As methionine is an essential amino acid for the synthesis of post-translational modification enzymes, a cross-expression system was developed utilizing separate promoters, allowing for the separate induction of target gene expression and biosynthetic enzymes. The amino acid replacement and incorporation efficiency of ncAAs into nisin derivatives was verified by matrix assisted laser desorption/ionisation time-of-flight analyzer (MALDI-TOF) and Liquid chromatography–mass spectrometry (LC-MS). Twelve nisin derivatives were purified by HPLC and their antimicrobial activity against two methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus faecalis*, vancomycin resistant *Enterococcus faecium*, *Bacillus cereus*, *L. monocytogenes*, *L. lactis* and *Micrococcus flavus* were investigated.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1. All *L. lactis* strains were grown in M17 broth supplemented with 0.5% (w/v) glucose at 30 °C for genetic manipulation. 5 μg/mL erythromycin and/or chloramphenicol were added when it was necessary. Chemical defined medium lacking tryptone (CDM-P)[26] was specially used for peptide expression and methionine analogues incorporation.

Table 1. Strains and plasmids used in this study.

Strains or Plasmids	Characteristics	References
Strains		
Lactococcus lactis NZ9000	*pepN::nisRK*; Expression host strain	[27]
Indicator strains		
Micrococcus flavus		Lab collection
Straphylococcus aureus CAL	Methicillin resistant (MRSA)	The University Medical Center Groningen, The Netherlands
Straphylococcus aureus MW2	Methicillin resistant (MRSA)	The University Medical Center Groningen, The Netherlands
Entercoccus faecium LMG 16003	Avaparicin and vancomycin resistant (VRE)	Laboratory of Microbiology, Gent, Belgium
Entercoccus faecalis LMG 16216	Vancomycin resistant (VRE)	Laboratory of Microbiology, Gent, Belgium
Bacillus cereus ATCC 14579		[28]
Listeria monocytogenes LMG 10470		[29]
Plasmids		
pIL3EryBTC	EryR, *nisBTC*, modification and transport of lantibiotics	[30]
pCZ-nisA	CmR, *nisA*, encoding NisA, under the control of P*czc*D promoter	[31]
pCZ-nisA-M17I	Point mutant of pCZ-nisA, with the Met 17 of nisin changed to Ile	This work
pCZ-nisA-M21V	Point mutant of pCZ-nisA, with the Met 21 of nisin changed to Val	This work
pCZ-nisA-M17I-M21V-M35	Point mutant of pCZ-nisA, with the Met 17 and 21 of nisin changed to Ile and Val, respecitviely, with Met 35	This work
pCZ-nisA-I4M-M17I-M21V	Point mutant of pCZ-nisA, with the Ile 4, Met 17 and Met 21 of nisin changed to Met, Ile and Val, respecitviely	This work
pNZnisP8H	CmR, *nisP*, encoding NisP mutant, with 8 histines	[32]

Table 2. Primers used in this study

Mutants	Primer	Sequence
M17I	pCZ-F	aacagtagtggcctcgtagc
	M17I-Rev	gctgttttcatgttacaaccaatcagagctcctgttttac
	M17I-Fwd	gtaaaacaggagctctgattggttgtaacatgaaaacagc
	pCZ-R	tagtctcggacattctgctc
M21V	pCZ-F	aacagtagtggcctcgtagc
	M21V-Rev	tacaatgacaagttgctgtttttacgttacaacccatcagagctc
	M21V-Fwd	agctctgatgggttgtaacgtaaaaacagcaacttgtcattgtag
	pCZ-R	tagtctcggacattctgctc
M35	NheI-For	atcagctagcacggaatagacatggtgttc
	M35-Rev1	ctacaatgacaagttgctgtttttacgttacaaccaatcagagctcctgttttac
	M35-Rev2	taccgcatgcctgcaggcttacattttgcttacgtgaatactacaatgacaagttg
I4M	NheI-For	atcagctagcacggaatagacatggtgttc
	I4-Rev1	cagagctcctgttttacaaccgggtgtacatagcgacatacttgtaatgcgtggtg
	I4-Rev2	acaatgacaagttgctgtttttacgttacaaccaatcagagctcctgttttac
	I4-Rev3	taccgcatgcctgcaggcttatttgcttacgtgaatactacaatgacaagttgctg

Construction of expression vectors

Molecular cloning techniques were performed following standard protocols.[33] The preparation of competent cells and transformation were performed according to Holo and Nes.[34] Fast digest restriction enzymes and ligase were used as recommended by the manufacturer. The nisin derivatives with one mutation in the core peptide (pCZ-nisA-M17I and pCZ-nisA-M21V) were produced by overlap extension PCR. For the construction of pCZ-nisA-M17I-M21V-M35 and pCZ-nisA-I4M-M17I-M21V, nested PCR of pCZ-nisA was used to introduce the mutation. The amplification was performed using Phusion Polymerase (Thermo Scientific) following the provider's instructions. After amplification and digestion with NheI and PaeI, it was ligated in pCZ-nisA digested with the same enzymes. The ligation product was desalted and transformed into *L. lactis* NZ9000. The plasmid was isolated and sequenced to check the integrity of the sequence.

Methionine analogues

The methionine analogue L-homopropargylglycine (Hpg) was purchased from Chiralix (Nijmegen, Netherlands). L-azidohomoalanine (Aha), L-norleucine (Nle), L-norvaline (Nva) and L-allyglycine (Alg) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). L-ethionine (Eth) was purchased from Alfa Aesar (Karlsruhe, Germany).

Precursor peptide precipitation

L. lactis strains harbouring pIL3eryBTC and pCZ-nisA were grown overnight in CDM-P with 5 µg/mL erythromycin and 5 µg/mL chloramphenicol. Subsequently, the overnight

culture was diluted in 20 mL fresh CDM-P back to OD600=0.1. When the OD600 reached 0.4~0.6, 10 ng/mL nisin was added to induce the expression of NisBTC. 3 h later, the cells were spun down at room temperature for 8 minutes at 5,000 rpm and then washed with CDM-P lacking methionine three times and re-suspended back in the initial volume of CDM-P lacking methionine. The medium was supplemented with either methionine (38 mg/L) or 50 mg/L methionine analogues, and 0.5 mM $ZnSO_4$ was added to express the peptides. After overnight growth, the supernatant was harvested by centrifugation at 8,500 rpm for 20 min at 4 °C. The precursor peptides were precipitated by Trichloroacetic acid (TCA) for further analysis according to Link et al.[35] Briefly, ice-cold 100% TCA solution was added to the supernatant to reach a final concentration of 10% TCA, and the solution was stored overnight at 4 °C. The precipitated peptide was pelleted by centrifugation at 8,000 rpm for 60 min at 4 °C. The supernatant was discarded and the pellet was washed with ice-cold acetone in half the original culture volume by a second centrifugation (8,000 rpm, 45 min, 4 °C). The acetone was discarded and the remaining acetone was evaporated off over several hours at room temperature. Dried pellets were suspended in 300 µl 0.05% aqueous acetic acid solution.

Tricine-SDS-PAGE analysis

The precipitated precursor peptides were analyzed by Tricine-SDS-PAGE according to Schagger et al.[36] 15 µl of each sample mixed with 4 µl loading dye was loaded on the gel. Coomassie brilliant blue G-250 was used to stain the gel.

LC-MS analysis of nisin derivatives

The precipitated precursor peptides were injected into the LC-MS system consisting of an Ultimate 3000 UHPLC system coupled via a HESI-II electrospray source with a Q-Exactive Orbitrap™-based mass spectrometer (all Thermo Scientific, San Jose, CA, USA). 3 µl of each samples were loaded onto a Kinetex EVO-C18 column (2.6 µm particles, 100 × 2.1 mm, Phenomenex). The eluents for the LC separation were (A) water and (B) Acetonitrile both containing 0.1% formic acid. The following gradient was delivered at a flow rate of 0.5 mL/min: 10% B until 1 min; then linear to 40% B in 9 min; linear to 80% B in 2 min; hold in 80% B for 2 min, after which a switch back to 10% B was performed in 0.1 min. After 5 min of equilibration the next injection was performed. The LC column was kept at 60 °C. The HESI-II electrospray source was operated with the parameters recommended by the MS software for the LC flow rate used (Spray voltage 3.5 kV (positive mode)); other parameters were sheath gas 50 AU, auxiliary gas 10 AU, cone gas 2 AU; capillary temperature 275 °C; heater temperature 400°C. The samples were measured in positive mode from m/z 500-2000 at a Resolution of 140,000 @ m/z 200. The instrument was calibrated in positive mode using the Pierce LTQ Velos ESI positive-ion calibration solution (Thermo Fisher Scientific,

Rockford, USA) (containing caffeine, the tetrapeptide MRFA and a mixture of fluorinated phosphazines ultramark 1621). The system was controlled using the software packages Xcalibur 4.1, SII for Xcalibur 1.3 and Q-Exactive Tune 2.9 (Thermo Fisher Scientific). The Xtract-algorithm within Xcalibur was used for deconvolution of the isotopically resolved data.

Purification of nisin and its derivatives

To obtain pure peptides for activity testing, the supernatant of 1 L culture was first incubated with purified NisP[32] at 37 °C for 3 h to cleave off the nisin leader, and then the supernatant was loaded on a C18 open column (Spherical C18, Sigma-Aldrich). The column was washed and eluted with different concentrations of buffer B (buffer A, Milli-Q with 0.1% TFA; buffer B, acetonitrile with 0.1% TFA). The active fractions were lyophilized and further purified by HPLC using an Agilent 1200 series HPLC with a RP-C12 column (Jupiter 4 um Proteo 90A, 250*4.6 mm, Phenomenex). The peak that is the fully modified peptide with the correct molecular weight was lyophilized and stored as powder until further use.

MALDI-TOF mass spectrometry characterization

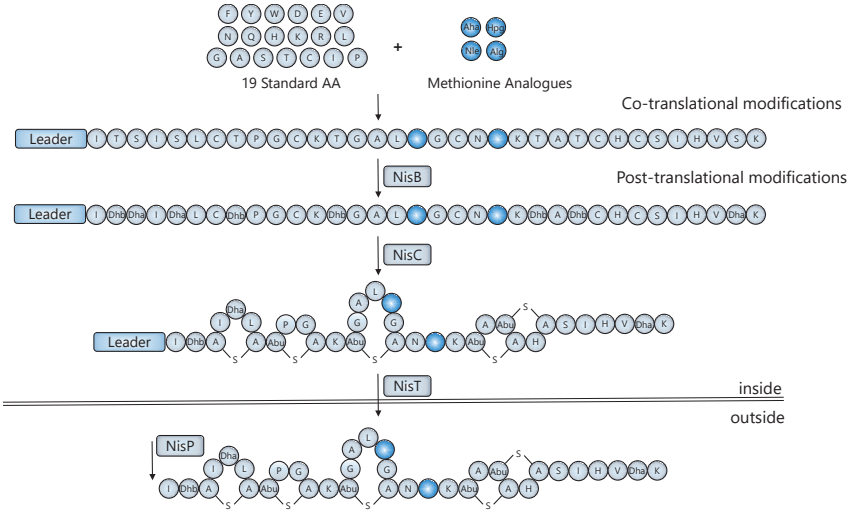
1 µL of each sample was spotted, dried and washed with Milli-Q water on the target. Subsequently, 1 µL of 5 mg/mL a-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) was spotted on top of the sample. An ABI Voyager DE Pro (Applied Biosystems) matrix-assisted laser desorption/ionization time-of-flight analyzer (MALDI-TOF) operating in linear mode using external calibration was used to obtain mass spectra.[30]

Agar well diffusion assay

Antimicrobial activity was tested against *M. flavus* according to protocols described previously.[30] 1 µg of sample was added to each well. The agar plate was incubated at 30 °C overnight, after which the zone of inhibition was measured.

Determination of the minimal inhibitory concentration (MIC)

HPLC purified and lyophilized peptides were resuspended in 0.05% aqueous acetic acid solution and the peptide amount was quantified by HPLC according to Schmitt et al.[26] For the MIC assay, the indicator strains CAL-MRSA, MW2-MRSA, *E. faecalis*, *E. faecium, B. cereus, L. monocytogenes and L. lactis* were first streaked on GM17 plate and cultured overnight. The peptide samples were diluted with 0.05% acetic acid to a concentration of 4–128 µg/mL (depending on the estimated activity of the peptide and the strain tested). GM17 broth was used for the activity test against *E. faecium, L. monocytogenes* and *L. lactis*. MHB was used for the activity test against CAL-MRSA, MW2-MRSA, *E. faecalis* and *B. cereus*. The MIC value test was performed according to Wiegand et al.[37]



Scheme 1. Incorporation of methionine analogues into nisin. 1. Co-translational modifications, insertion of methionine analogues into precursor peptide. 2. Post-translational modifications, converting the linear precursor peptide into an active polycyclic peptide.

Results

A cross expression system to incorporate Met analogues into nisin in *L. lactis*

Until now, the Gram-negative *Escherichia coli* is the only prokaryotic expression host used for the incorporation of methionine analogues into proteins. Here, the Gram-positive expression host *L. lactis* is used for the incorporation of methionine analogues into the lantibiotic nisin. After ribosomal synthesis of the precursor peptide with 19 standard amino acids and with various methionine analogues, the unmodified prenisin is processed by its dedicated modification machinery (Scheme 1).

As methionine is an essential amino acid for the expression of post translational modification (PTM) enzymes, a cross expression system which allows for the expression of nisin derivatives and PTM enzymes at different times was used for this study. *L. lactis* NZ9000 was transformed with a plasmid encoding the expression of NisBTC under the control of Pnis promoter and the other plasmid encoding the expression of nisin derivatives was controlled by the PczcD promoter. The expression of NisBTC was first conducted with the supplementation of methionine, and then the medium was replaced by new medium containing methionine analogues to express the peptides (Figure 1A).

Production of nisin and its derivatives

To test the effect of single methionine analogues replacement of nisin, four single Met mutants, i.e. M17I, M21V, M17I-M21V-M35, and I4M-M17I-M21V were constructed

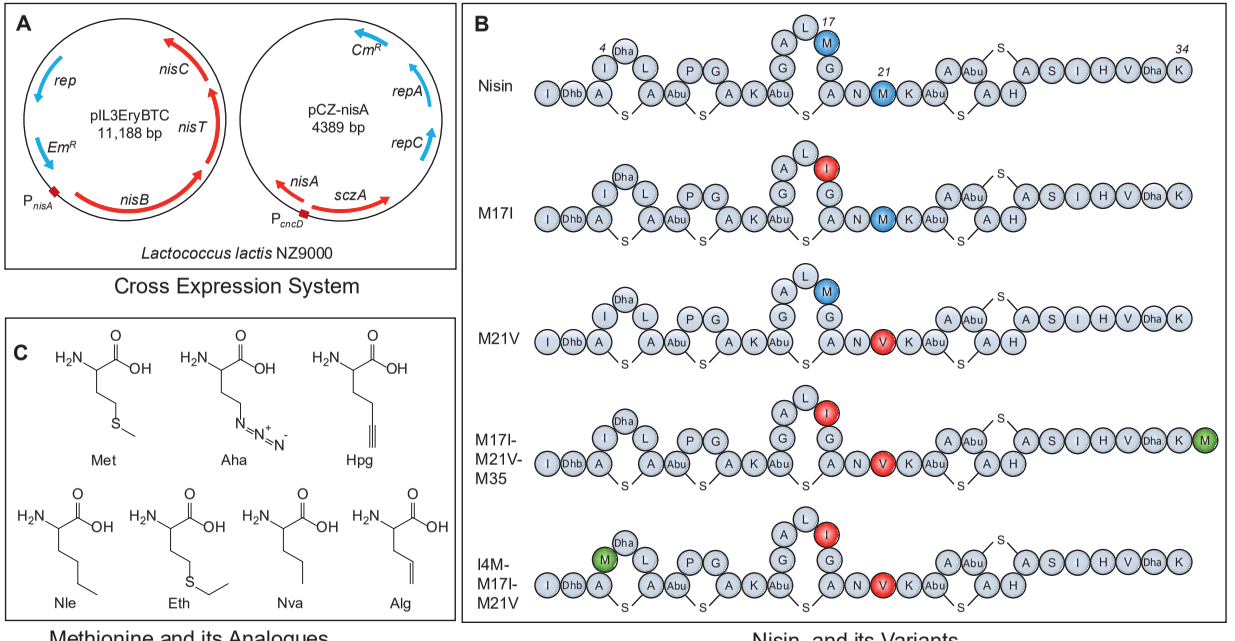


Figure 1. (A) A cross expression system with two plasmids. *SczA*, encoding the repressor of P*czcD*; P*czcD*, a zinc inducible promoter; *nisA*, encoding NisA; *repA* and *repC*, encoding plasmid replication proteins; *cmR*, chloramphenicol resistance gene; P*nisA*, a nisin inducible promoter; *nisB*, encoding NisB; *nisT*, encoding NisT; *nisC*, encoding NisC; *EmR*, erythromycin resistance gene. (B) Peptide sequence of nisin and nisin derivatives. Dha, dehydroalanine; Dhb, dehydrobutyrine; A-S-A, lantionine; Abu-S-A, methyllanthionine; Ile4, Met17, Met21, and Met35 were mutated. In blue, wild-type Met positions; In green, Met residues replaced by Ile or Val, In red, Met residues at novel positions. (C) Structures of methionine and its analogues. Met, L-methionine; Aha, L-azidohomoalanine; Hpg, L-homopropargylglycine; Nle, L-norleucine; Eth, L-ethionine; Nva, L-norvaline; Alg, L-allyglycine.

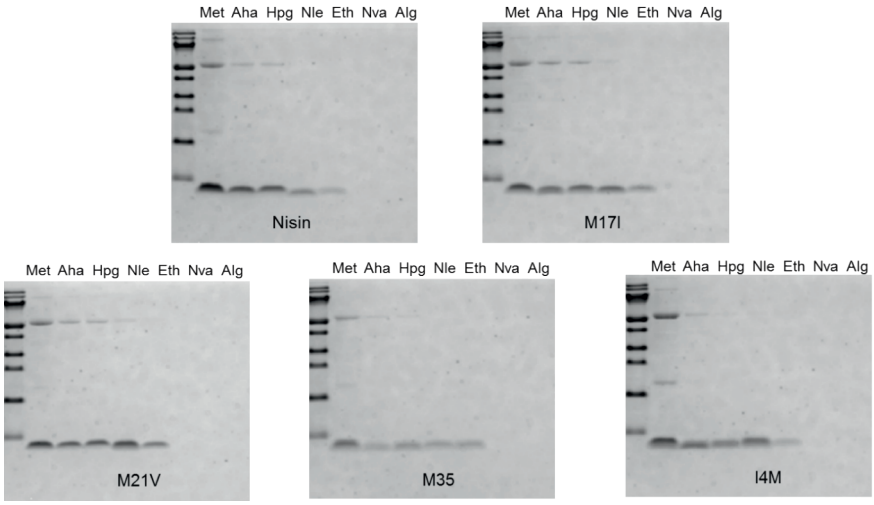


Figure 2. Coomassie-blue stained Tricine-SDS-PAGE gel. Each well contained TCA-precipitated prepetides from 1 mL supernatant.

Table 1. The incorporation efficiency of nisin and its derivatives analysed by LC-MS.

Peptide		Incorporation efficiency
Nisin	Aha	88%
	Hpg	87%
	Nle	77%
	Eth	56%
M17I	Aha	96%
	Hpg	92%
	Nle	88%
	Eth	71%
M21V	Aha	99%
	Hpg	91%
	Nle	88%
	Eth	73%
M17I-M21V-M35	Aha	>99.5%
	Hpg	>99.5%
	Nle	51%
	Eth	71%
I4M-M17I-M21V	Aha	95%
	Hpg	93%
	Nle	88%
	Eth	71%

>99.5% means the peak of peptides containing methionine is undetectable. The incorporation efficiency indicates the percentage of the produced peptide with methionine analogues incorporated.

Table 2. MS analysis of prenisin and its derivatives.

Peptide	Methionine Analogue	Modification	Predicted Mass (Da)		Measured Mass (Da)									
					Met		Aha		Hpg		Nle		Eth	
			+Met1	-Met1	+Met1	-Met1	+Met1	-Met1	+Met1	-Met1	+Met1	-Met1	+Met1	-Met1
Nisin	Met	-8H_2O	5818.85	5687.66	5818.80	5687.76								
		-8H_2O+Oxi	5834.85	5703.66	5835.80	5704.76								
		-8H_2O+2Oxi	5850.85	5719.66	5850.79	5720.75								
	Aha	-8H_2O	5803.63	5677.51			5803.84	5677.79						
	Hpg	-8H_2O	5752.66	5643.53					5752.84	5643.79				
	Nle	-8H_2O	5764.75	5651.59							5764.93	5651.85		
		-7H_2O	5782.77	5669.61							5782.90	5669.81		
	Eth	-8H_2O	5860.93	5715.71									5861.84	5716.79
		-8H_2O+Oxi	5876.93	5731.71									5877.84	5732.79
		-8H_2O+2Oxi	5892.93	5747.71									5893.83	5748.78
M17I	Met	-8H_2O	5800.81	5669.62	5800.85	5669.81								
		-8H_2O+Oxi	5816.81	5685.62	5816.84	5685.80								
	Aha	-8H_2O	5790.66	5664.54			5790.87	5664.82						
	Hpg	-8H_2O	5756.68	5647.55					5756.87	5647.82				
	Nle	-8H_2O	5764.74	5651.58							5764.93	5651.85		
		-7H_2O	5782.76	5669.60							5782.90	5669.82		
	Eth	-8H_2O	5828.86	5683.64									5829.87	5683.82
		-8H_2O+Oxi	5844.86	5699.64									5845.87	5700.82
		-8H_2O+2Oxi	5860.86										5861.87	
M21V	Met	-8H_2O	5786.79	5655.60	5786.83	5655.79								
		-8H_2O+Oxi	5802.79	5671.60	5802.82	5672.78								
	Aha	-8H_2O	5776.64	5650.52			5776.85	5650.80						
	Hpg	-8H_2O	5742.66	5633.53					5742.85	5633.50				
	Nle	-8H_2O	5750.72	5637.56							5750.91	5637.83		
		-7H_2O	5768.74	5655.58							5768.89	5655.81		
	Eth	-8H_2O	5814.84	5669.62									5815.85	5670.80
		-8H_2O+Oxi	5830.84	5685.62									5831.85	5685.80
		-8H_2O+2Oxi	5846.84										5846.85	

Peptide	Methionine Analogue	Modification	Predicted Mass (Da)		Measured Mass (Da)									
					Met		Aha		Hpg		Nle		Eth	
			+Met1	-Met1	+Met1	-Met1	+Met1	-Met1	+Met1	-Met1	+Met1	-Met1	+Met1	-Met1
M17I-M21V-M35	Met	-8H_2O	5899.95	5768.76	5899.91	5768.87								
		-7H_2O	5917.97	5786.77	5917.81	5786.88								
		-7H_2O+Oxi	5933.97	5802.77	5933.91	5802.87								
	Aha	-8H_2O	5889.80	5763.68			5889.94	5763.89						
		-7H_2O	5907.82	5781.70			5906.94	5781.89						
	Hpg	-8H_2O	5855.82	5746.69					5855.97	5746.88				
		-7H_2O	5873.84	5764.71					5873.94	5764.89				
	Nle	-8H_2O	5863.88	5750.72							5864.00	5750.91		
		-7H_2O	5881.90	5768.74							5882.01	5768.93		
	Eth	-8H_2O	5928.00	5782.78									5928.94	5782.89
		-7H_2O	5946.02	5800.80									5945.94	5800.89
		-7H_2O+Oxi	5962.02	5816.80									5961.94	5816.89
I4M-M17I-M21V	Met	-8H_2O	5786.79	5655.60	5787.83	5655.79								
		-7H_2O	5804.81	5673.61	5804.82	5672.78								
		-7H_2O+Oxi	5820.81	5689.61	5819.82	5689.78								
	Aha	-8H_2O	5776.64	5650.52			5776.85	5650.80						
		-7H_2O	5794.66				5792.85							
	Hpg	-8H_2O	5742.66	5633.53					5742.85	5633.80				
		-7H_2O	5760.68						5758.85					
	Nle	-8H_2O	5750.72	5637.56							5750.91	5637.83		
		-7H_2O	5768.74	5655.58							5767.90	5655.81		
	Eth	-8H_2O	5814.84	5669.62									5815.85	5670.80
		-8H_2O+Oxi	5830.84	5685.62									5830.85	5686.80
		-8H_2O+2Oxi	5846.84										5846.84	

+Met1,with N-terminal Met; -Met1, without N-terminal Met; -8H_2O, eight times dehydrated; -8H_2O+Oxi, eight times dehydrated and one time oxidized; -8H_2O+2Oxi, eight times dehydrated and two times oxidized; -7H_2O, seven times dehydrated; -7H_2O+Oxi, seven times dehydrated and one time oxidized.

(Figure 1B). Six methionine analogues, i.e. Aha, Hpg, Nle, Eth, Nva, and Alg were selected for the incorporation (Figure 1C). These mutants were used to evaluate the incorporation efficiency of the methionine analogues at the different positions and investigate the antimicrobial activity of these nisin derivatives with methionine analogues incorporated.

The expression level of nisin and nisin derivatives is shown in Figure 2. The protein quantities in the first five lanes showed that Aha, Hpg, Nle, and Eth can be incorporated into nisin and its derivatives at varying levels. However, incorporation of Nva and Alg was not observed at any moment. Nisin and its derivatives showed the highest production yield when normal methionine was supplemented. Effectively, a lower production yield was observed in the presence of methionine analogues, Eth in particular. Additionally, a drastically lower yield was observed overall for the mutant M17I-M21V-M35, when compared to all other constructs. To assess the presence of post translational modifications and incorporation of ncAAs, all samples were further analyzed by HPLC and MALDI-TOF. The resulting spectra showed that the production yield of nisin and its derivatives with Aha and Hpg are much higher than the ones with Nle and Eth. The methionine analogues Nle and Eth have a negative influence on the dehydration rate, as large fractions of 7 times dehydrated peptides were observed. Surprisingly, the production yield of fully modified M21V is even higher than that of WT nisin. Compared to the WT and the other two derivatives, M17I-M21V-M35 and I4M-M17I-M21V showed much lower production yields.

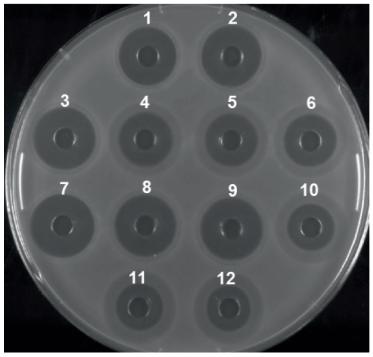
LC-MS analysis of nisin derivatives

In order to estimate the efficiency of methionine analogue incorporation, the precipitated precursor peptides were subjected to liquid chromatography-electrospray mass spectrometry (LC-MS). The LC-MS data showed that the incorporation efficiency of Aha and Hpg into mutants M17I, M21V, and I4M-M17I-M21V were more than 91%, while the incorporation efficiency of Nle and Eth were 88% and 71-73%, respectively. The incorporation efficiencies of Aha and Hpg into M17I-M21V-M35 were at least 99.5%; the peaks of peptides containing methionine were undetectable. However, the incorporation efficiencies of Nle and Eth were only 51% and 71%, respectively. In the case of nisin, the incorporation efficiency was 88% for Aha, 87% for Hpg, 77% for Nle and 56% for Eth. Generally, the incorporation efficiency of ncAAs declined in the order Aha > Hpg > Nle >Eth (Table 1).

Incorporation of Aha and Hpg into nisin, M17I, or M21V wouldn't affect the dehydration efficiency, as peptides with 7 times dehydrated residues were almost undetectable. However, introducing Nle and Eth resulted in a large fraction of peptides containing 7 times dehydration. The dehydration of M17I-M21V-M35 and I4M-M17I-M21V was dramatically affected by the mutation. Both with methionine or methionine

Table 3. MS analysis of nisin and its derivatives used for activity test

Peptide	Predicted Mass (Da)	Measured Mass (Da)
Nisin	3354.09	3354.02
M17Aha-M21Aha	3343.94	3343.77
M17Hpg-M21Hpg	3309.96	3309.16
M17I	3336.05	3335.46
M17I-M21Aha	3330.98	3330.14
M17I-M21Hpg	3313.99	3313.45
M21V	3322.03	3321.60
M21V-M17Aha	3316.96	3316.78
M21V-M17Hpg	3299.97	3299.81
M17I-M21V-M35	3435.18	3434.93
M17I-M21V-M35Aha	3430.11	3429.93
M17I-M21V-M35Hpg	3413.12	3413.07



Micrococcus flavus

	Peptide	Diameter
1	Nisin	22.0 mm
2	M17Aha-M21Aha	23.0 mm
3	M17Aha-M21Aha	23.9 mm
4	M17I	20.4 mm
5	M17Aha-M21Aha	19.8 mm
6	M17Aha-M21Aha	20.1 mm
7	M21V	24.5 mm
8	M21V-M17Aha	23.6 mm
9	M21V-M17Hpg	24.0 mm
10	M17I-M21V-M35	18.0 mm
11	M17I-M21V-M35Aha	17.8 mm
12	M17I-M21V-M35Hpg	16.8 mm

Figure 3. Antimicrobial activity of nisin and its derivatives against *M. flavus*. Grey: values that are improved in comparison to nisin.

analogues, the extent of dehydration in the peptides varied (Data not shown). Additionally, methionine and ethionine can be oxidized, and peaks corresponding to oxidized products were indeed observed. Furthermore, the first methionine of prenisin is usually cleaved by the enzyme methionine aminopeptidase (MAP). However, a large portion of precursor peptide produced by this system contained the N-terminal Met. The molecular weight of both peaks is shown in Table 2.

Antimicrobial activity of nisin and its derivatives

Considering the production yield of fully modified peptides, 12 peptides were selected to be purified at large scale (Table 3) and their antimicrobial activities were investigated (Figure 3). *M. flavus* was used as an indicator strain in an agar-well diffusion assay to assess the antimicrobial activity. The results showed that M17Aha-M21Aha, M17Hpg-M21Hpg, M21V, M21V-M17Aha, and M21V-M17Hpg have higher antimicrobial

Table 4. MIC values (μM) of nisin and its derivatives.

Peptide	CAL-MRSA	MW2-MRSA	*B. cereus*	*E. faecalis*	*E. faecium*	*L. monocytogenes*	*L. lactis*
Nisin	10.39	5.19	5.19	2.60	0.32	2.60	0.020
M17Aha-M21Aha	19.99	13.33	6.66	3.33	0.42	1.67	0.026
M17Hpg-M21Hpg	19.41	19.41	9.70	4.85	0.61	4.85	0.019
M17I	>19.92	>19.92	>19.92	19.92	2.49	4.98	0.622
M17I-M21Aha	>17.42	>17.42	8.71	17.42	2.18	4.36	0.544
M17I-M21Hpg	>19.08	>19.08	19.08	19.08	2.38	4.77	0.596
M21V	9.81	2.45	4.90	4.90	0.61	2.45	0.019
M21V-M17Aha	>19.72	19.72	19.72	9.86	0.62	4.93	0.039
M21V-M17Hpg	>19.74	19.74	19.74	9.87	0.62	4.93	0.019
M17I-M21V-M35	>18.33	>18.33	>18.33	>18.33	2.29	>18.33	0.573
M17I-M21V-M35Aha	>19.96	>19.96	>19.96	>19.96	2.49	>19.96	0.624
M17I-M21V-M35Hpg	>16.82	>16.82	>16.82	16.82	2.10	>16.82	>0.526

Grey: MIC values that are improved in comparison to nisin.

activity compared to WT nisin and mutant M21V had the best activity. However, in all the mutants of M17I and M17I-M21V-M35, antimicrobial activity decreased dramatically.

The MIC values were determined for *L. lactis* and six Gram-postive pathogenic strains. The tested strains included two Staphylococci, two Enterococci, *B. cereus* and *L. monocytogenes* (Table 4). Compared to nisin, M21V showed higher activity against *S. aureus*, *B. cereus*, *L. monocytogenes* and *L. lactis* but decreased activity against both Enterococcus strains. M17Aha-M17Hpg showed improved activity against *L. monocytogenes* but activity against other strains were strongly reduced. M17Hpg-M21Hpg and M21V-M17Hpg displayed a slightly increased activity against *L. lactis* but activity against other strains was strongly reduced compared to nisin.

Discussion

By incorporating ncAAs with different structures and properties, the diversity of ribosomally produced peptides can be dramatically increased. This can be achieved by introducing amino acids containing atoms or functional groups as side chains not occurring in nature, e.g. fluorine and azide.[18,19,38-42] Incorporation of ncAAs offers unique physicochemical properties over conventional peptide mutagenesis.[6,11] It provides greatly enhanced structural and functional diversity, while retaining or even improving activity. As *L. lactis* is auxotrophic for methionine and methionine is

essential for the expression of modification enzymes, a cross expression system was used. The combination of nisin- and zinc-inducible gene expression systems allows for the expression of modification enzymes and peptides at different time. Although the expression of the modification machinery NisBTC was induced in advance, no effect on modification efficiency was observed.

Four nisin mutants were constructed to incorporate methinonine analogues. Six methionine analogues were installed at four different positions, and each combination was tested. In total, 20 novel ncAA containing nisin derivatives were produced and identified. The production yield of each derivative depended on the methionine position and what analogue was used. Methionine analogue incorporation in nisin, M17I and M21V gave higher yields of fully modified peptides than M17I-M21V-M35 and I4M-M17I-M21V, which may be due to the intolerance of the modification machinery to a change of chemical structure at sites I4 and M35. Surprisingly, mutant M21V shows even a higher production yield than that of nisin. Aha, Hpg, Nle, and Eth can be incorporated successfully into nisin and all four mutants. However, incorporation of Nva and Alg was not observed, and addition of these analogues to a culture lacking methionine leads to arrested cell growth. These results strongly indicate these amino acids cannot be incorporated by *L. lactis*. This may be due to the fact that these ncAAs could not be recognized by lacMetRS. The incorporation efficiency of ncAAs declined in the order Aha > Hpg > Nle > Eth. LC-MS analysis showed 90-100% substitution of methionine by Aha and Hpg, suggesting they are excellent methionine surrogates. It may be due to the rate of activation of Aha and Hpg by methionyl-tRNA synthetase (MetRS) during translation, which finally results in the higher yield and efficient modification. However, the integration speed of Nle and Eth during translation is relatively slow which leads to a lower yield and insufficient modification.

In consideration of the production yield of fully modified peptides, 12 peptides were purified in large scale for antimicrobial activity test. The results showed that the replacement of Met with Met analogues with different properties can alter the antimicrobial activity and spectrum. M21V has been reported to have enhanced bioactivity and specific activity against all tested Gram-positive pathogens including four VRE strains compared to WT nisin.[43, 44] In our study, M21V showed reduced activities against two enterococci strains, but retained a high activity against others. Peptides like M17Aha-M21Aha showed strongly reduced activities against several strains, but retained a high activity against specific strains, suggesting an antimicrobial spectrum changed. Improving the antimicrobial activity of nisin turned out to be difficult. However, engineering nisin can generate new nisin derivatives which have different properties and can be used for specific targets. In addition, some nisin derivatives showed different inhibition activity in solid media tests compared to the broth MIC test. This phenomenon can be related to the difference in diffusion ability.

Taken together, we have demonstrated for the first time the incorporation of methionine analogues into RiPPs in *L. lactis*. Four methionine analogues were successfully installed into four distinct positions of the lantibiotic nisin. The genetic code of *L. lactis* can be regarded to be expanded by incorporating methionine analogues. The structural diversity was enhanced while retaining or even improving antimicrobial activity against specific pathogens or Gram-positive bacteria. These results further confirm the possibility of incorporating ncAAs with diverse structure and properties into RiPPs. In addition, replacement of methionine by analogues that possess unique chemical reactivity (e.g. azide and alkyne) offers the potential for post-translational protein modification, i.e. they can be used as chemical handles for click chemistry coupled with a variety of ligands such as fluorophores, glycans, PEGs, lipids, peptide moieties and other antimicrobial moieties. The insertion of ncAAs during translation along with the possibility for their subsequent modification (post-synthetic conjugation) will expand the chemical and functional space of RiPPs.

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CHAPTER 5

Synthesis of nisin conjugates via click chemistry and their characterization

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Submitted

Abstract

Coupling the antimicrobial peptide nisin with specific functional moieties and semi-synthetic fragments of nisin offers exciting opportunities to produce novel derivatives with desirable properties for new and improved functions and applications. Here, two methods are employed to obtain conjugates of nisin and several of its derivatives via click chemistry. In the first approach, azidopropylamine was added to the C-terminus of several truncated variants of nisin through *in vitro* addition. Subsequently, these variants were conjugated to five synthetic hydrophobic pentynoyl peptides. The resulting semi-synthetic nisin analogues displayed potent inhibition of bacterial growth. The second method utilizes reactive side chains (i.e. alkyne and azide), installed into nisin derivatives as residues of non-canonical amino acids (ncAAs) through force feeding. These reactive groups, so called chemical handles, can be used to conjugate nisin with e.g. peptide moieties and fluorescent probes. Six different dimeric nisin constructs, three nisin hybrids and six fluorescently labeled nisin variants were prepared using this approach. All resulting compounds retained antimicrobial activity, which substantiates the potential of this method as a tool to further study the localization and mode of action of nisin. The success of both approaches in creating viable conjugates of nisin and its derivatives, encourages further exploring the use of additional modules, e.g. active moieties of lanthipeptides, fluorescent groups, antimicrobial moieties and other relevant groups.

Keywords: click chemistry, azidohomoalanine, homopropargylglycine, dimeric nisin, nisin hybrids, fluorescently labeled nisin, nisin analogues

Introduction

Nisin is the first discovered and the best studied lanthipeptide. It is produced by *Lactococcus lactis* and has potent activity against a broad spectrum of Gram-positive bacteria, including many antibiotic-resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococcus (VRE).[1,2] Due to its potent antimicrobial activity and low toxicity to humans, it is frequently used as a food preservative.[3] Nisin contains one lanthionine and four methyllanthionine rings and has a dual mode of action. The first two rings (AB) form the lipid II recognition site. By binding to the peptidoglycan precursor lipid II, nisin inhibits cell wall biosynthesis. The last three rings (CDE), which include the hinge region, constitute the membrane insertion domain. After rings AB dock to lipid II[4], rings CDE can insert into the bacterial membrane to create pores.[5] Nisin's unique mode of action and potent antimicrobial activity would make it an attractive candidate for development into an antibiotic. However, its proteolytic degradation in the gut precludes oral delivery and instability of the dehydroresidues limits the possible therapeutic application of the full-length peptide by injection. Coupling moieties to nisin and semi-synthetic fragments of nisin have led to the development of nisin derivatives with enhanced antimicrobial activity against clinically relevant bacterial pathogens.[6]

One of the most widely used tools to achieve peptide coupling is click chemistry.[7] It is referred to as "copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC)" and is a region-selective copper (I) catalytic cycloaddition reaction between an azide and an alkyne that give rise to a triazole.[8] Due to its high level of reliability, specificity, biocompatibility, easiness to perform, and mild reaction conditions, click chemistry is being used increasingly in diverse areas, such as bioconjugation, drug discovery and polymer science.[9-11] Peptide coupling using click chemistry has been the subject of several studies for the development of target-specific bacterial probes and expanding its application.[6,12-17] The most prominent example is the coupling of nisin AB to lipid moieties rendering the resulting hybrids with superior stability and potent antimicrobial activities against drug-susceptible and -resistant strains of Gram-positive bacteria.[6]

Here, two sets of experiments were designed for the synthesis of nisin conjugates. In the first set of experiments, C-terminally functionalized nisin AB-azide and nisin ABC-azide are coupled with five hydrophobic pentynoyl peptides. Hypothetically, the lipid-II targeting structure of nisin could guide the conjugate to the bacterial membrane, where the hydrophobic tail would flip into the membrane core and anchor the conjugate tightly to the membrane. Concurrently, this setup allows for insight into the possibility of creating semi-synthetic nisin analogues that retain antimicrobial activity, while imparting improved stability. The second set involves coupling of nisin derivatives (M17I-M21Aha, M21V-M17Aha, M17I-M21V-M35Aha, M17I-M21V-M35Hpg,

M21V-M17Hpg, and M17I-M21Hpg) either mutually or with nisin ABC-azide, Cy5-azide, and 6-FAM-alkyne. These coupling reactions afford six dimeric nisin constructs, three nisin hybrids and six fluorescently labeled nisin variants, which are applicable for studying nisin's localization and mode of action.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this work are given in Table 1. Chemical defined medium lacking tryptone (CDM-P)[18] was specially used for peptide purification supplemented with 5 μg/mL erythromycin and 5 μg/mL chloramphenicol.

Table 1. Strains and plasmids used in this work.

Strains or Plasmids	Characteristics	References
Strains		
Lactococcus lactis NZ9000	*pepN::nisRK*; Expression host strain	[19]
Indicator strains		
Micrococcus flavus		Lab collection
Straphylococcus aureus MW2	Methicillin resistant (MRSA)	The University Medical Center Groningen, The Netherlands
Entercoccus faecium LMG 16003	Avaparicin and vancomycin resistant (VRE)	Laboratory of Microbiology, Gent, Belgium
Listeria monocytogenes LMG 10470		[20]
Plasmids		
pIL3EryBTC	EryR, *nisBTC*, modification and transport of lantibiotics	[21]
pCZ-nisA	CmR, *nisA*, encoding NisA, under the control of P*czc*D promoter	[22]
pCZ-nisA-M17I	Point mutant of pCZ-nisA, with the Met 17 of nisin changed to Ile	Chapter 4
pCZ-nisA-M21V	Point mutant of pCZ-nisA, with the Met 21 of nisin changed to Val	Chapter 4
pCZ-nisA-M17I-M21V-M35	Point mutant of pCZ-nisA, with the Met 17 and 21 of nisin changed to Ile and Val, respecitviely, with Met 35	Chapter 4
pNZnisP8H	CmR, *nisP*, encoding NisP mutant, with 8 histines	[23]

Purification of nisin derivatives that contain Aha or Hpg moieties

The expression strain NZ9000 with appropriate plasmids was first cultured overnight in CDM-P with 5 μg/mL erythromycin and 5 μg/mL chloramphenicol and then diluted into fresh CDM-P back to OD600=0.1. When OD600 reached 0.4~0.6, 10 ng/mL nisin was added to induce the expression of NisBTC. Cells were harvested 3 hours

after induction by centrifugation (5000 rpm, 8 min) at room temperature and washed three times with CDM-P lacking methionine and then re-suspended back in the initial volume of CDM-P lacking methionine supplemented with 50 mg/L azidohomoalanine (Aha) or homopropargylglycine (Hpg). In the mean time, 0.5 mM $ZnSO_4$ was added to express the peptides. The supernatant was harvested after overnight growth by centrifugation (8,500 rpm, 20 min) at 4 °C and then incubated with purified NisP at 37 °C for 3 hours to cut off the nisin leader and finally loaded on a C18 open column (Spherical C18, Sigma-Aldrich). The active fractions were further purified by HPLC and the active peaks were analyzed by MALDI-TOF. The peak which contains the fully modified peptide with the correct mass was lyophilized and stored as powder until further use.

Preparation of nisin AB-azide

Nisin (180 mg) was dissolved in 150 mL Tris buffer (25 mmol NaOAc, 5 mmol Tris acetate, 5 mmol $CaCl_2$, pH7.0) and the solution was cooled on ice for 15 min. Subsequently, trypsin (15 mg) was added and warmed up to room temperature for 15 min. The reaction was performed at 30 °C for 16 h and an extra 15 mg trypsin was added. After 24 h incubation, another 15 mg trypsin was added and incubated for another 24 h. The reaction mixture was acidified with HCl (1 M) to pH 4.0 followed by adding 3 mL MeCN and concentrated *in vacuo*. The pure nisin AB was purified from the mixture by RP-HPLC and lyophilized to obtain a white powder (20 mg). Nisin AB (10 mg, 8.6 µmol) was dissolved in dimethylformamide (DMF) (100 µl) and azidopropylamine (44 µl, 43.2 mg, 432 µmol), BOP ((benzotriazol-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate) (7.6 mg, 17.2 µmol) or PyBOP (Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) (9 mg, 17.2 µmol), and DIPEA (N,N-diisopropylethylamine) (6 ul, 34.8 µmol) were added. The reaction was vortexed for 20 min and subsequently quenched with 5 mL buffer (H_2O : MeCN, 95:5 + 0.1% TFA). The reaction mixture was purified by HPLC and pure nisin AB-azide was lyophilized to obtain a white powder (8 mg).

Preparation of nisin ABC-azide

Nisin (180 mg) was dissolved in 150 mL Tris buffer (25 mmol Tris acetate, pH7.5) and the solution was cooled on ice for 15 min. Then α-chymotrypsin (15 mg) was added and stirred at room temperature for 15 min. The enzymatic digestion was performed same as described for nisin AB. Nisin ABC was purified from the mixture by RP-HPLC and then lyophilized to obtain a white powder (20 mg). Nisin ABC (10 mg, 6.5 µmol) was dissolved in DMF (50 µl). The azide-coupling reaction was performed same as described for nisin AB.

Preparation of the hydrophobic pentynoyl peptides

The pentynoyl peptides were prepared using a manual Fmoc-based solid-phase peptide synthesis scheme. The sequences were grown on 2-clorotrityl resins pre-loaded with either Fmoc (fluorenylmethyloxycarbonyl)-Oic or Fmoc-Lys(Boc)-OH. The resin loading was estimated at 0.7–0.8 mmol/g. The synthesis was performed in DMF using the Fmoc-amino acid (2.5 equiv.) pre-activated with the TBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate) / HOBt mixture (2.4 / 2.5 equiv.) under addition of DIPEA (5 equiv.). The N-terminal pentynoyl moiety was installed under coupling with pentynoic acid under same activation conditions. The Fmoc group was removed by treatment with 22 vol% piperidine in DMF. The final peptides were cleaved off the resin by treatment with hexafluoroisopropanol : dichloromethane (1:3, vol : vol) mixture. The peptides were additionally purified on short silica gel columns using dichloromethane-methanol gradient elution. Pentynoyl-(Oic)9-OH peptide was additionally purified by precipitation from methanol. The Boc-group was removed from the lysine side-chains by treatment with 4 M hydrogen chloride in dioxane. The identity and purity of the final peptides were confirmed by mass-spectra (ESI-Orbitrap) and ^{1}H-NMR spectra (CD_3OD, 700 MHz). The peptides were obtained in 10–50 mg quantities. For extended discussion on the peptide preparation and properties see[24].

Click chemistry

A stock solution of $CuSO_4$ (10 mg, 100 mM), BTTAA (2-(4-((bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)-acetic acid) (25 mg, 50 mM), THPTA (tris((1-hydroxy-propyl-1H-1,2,3-triazol-4-yl)methyl)amine) (25 mg, 250 mM) and sodium ascorbate (200 mg, 1 M) in ddH_2O and a stock solution of O3 (1 mg, 36 mM), O6 (1.8 mg, 36 mM), O9 (2.6 mg, 36 mM), O3K3 (1.9 mg, 36 mM) and O6K3 (2.7 mg, 36 mM) in DMF (50 µl) were prepared, aliquoted and stored at −20 °C for further use. Firstly, nisin AB-azide (25 µg, 0.02 µmol) or nisin ABC-azide (40 µg, 0.02 µmol) were dissolved in 100 mM phosphate buffer (pH7.0, final reaction volume: 200 µl). Then, the appropriate O3, O6, O9, O3K3, or O6K3 (5 ul, 0.18 µmol) and $CuSO_4$ (4 µl, 0.4 µmol) : THPTA (8 µl, 2 µmol) or BTTAA (40 µl, 2 µmol)-premix were added followed by the addition of sodium ascorbate (20 µl, 20 µmol). The reaction was performed at 37 °C for 1 h and purified directly by RP-HPLC. The pure product-containing fractions were lyophilized to obtain nisin conjugates **6–15** as white fluffy powder. The reaction was further scaled up in ratio to obtain more products. Besides, M17I-M21Aha, M17I-M21Hpg, M17I-M21V-M35Aha, M17I-M21V-M35Hpg, M21V-M17Aha, and M21V-M17Hpg (70 µg, 0.02 µmol) were reacted either mutually or with nisin ABC-azide (40 µg, 0.02 µmol), Cy5-azide (5 µl, 10 mg/mL) and 6-FAM-alkyne (4 µl, 10 mg/mL) as described above to obtain six dimeric nisin constructs, three nisin hybrids and six fluorescently labeled nisin variants.

Agar well diffusion assay

Agar well diffusion assay against *Micrococcus flavus* was performed as described previously.[21] 0.15 nmol of each sample was added to each well. The agar plate was incubated at 30 °C overnight, after which the zone of inhibition was measured.

Determination of the minimal inhibitory concentration (MIC)

All samples were resuspended in 0.05% aqueous acetic acid solution and the peptide amount was quantified by HPLC according to Schmitt et al.[18] The indicator strains MW2-MRSA, *E. faecium, L. monocytogenes and L. lactis* were first streaked on GM17 plate and cultured overnight. The peptide samples were diluted with 0.05% acetic acid to a concentration of 40-320 μM (depending on the estimated activity of the peptide and the strain tested). The MIC value test was performed according to Wiegand et al.[25]

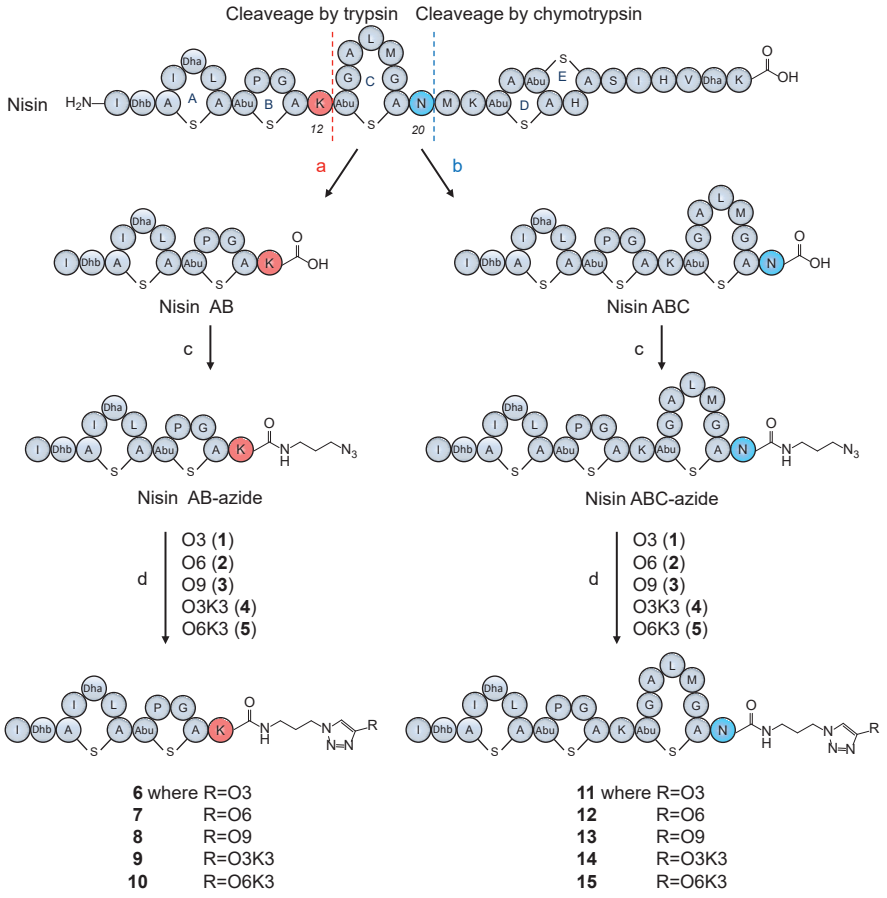
Fluorescence microscopy

Cultures of overnight grown *E. faecium* were diluted 1:100 and incubated in GM17 at 37 °C for about 4 h to reach OD600 of 0.5. Then, 0.5 mL of culture were centrifuged at 7000 rpm for 3 min. Fluorescently labeled nisin variants were added into the Eppendorf tube with cells at desired concentration in 100 μl saline solution and cells were incubated at 37 °C for 30 min. After three other washes in saline buffer, 0.6 μl bacterial suspensions and 1% low-melting-point agar were added to a microscopy plate and the localization of nisin variants were inspected with a Delta Vision Elite inverted epifluorescence microscope (Applied Precision, GE Healthcare, Issaquah, WA, USA) equipped with a stage holder, a climate chamber, a seven-color combined set InsightSSI Solid-state Illumination module and an sCMOS camera (PCO AG, Kelheim, Germany). Excitation was set to 646 nm and emission to 662 nm to capture Cy5-azide fluorescence. For 6-FAM-alkyne fluorescence, we employed 490 nm for excitation and emission at 513 nm. Images were obtained by ImageJ software.

Results

Semi-synthetic nisin AB and nisin ABC conjugates

Nisin was digested using trypsin and chymotrypsin to generate nisin-AB and nisin-ABC, respectively (A, B and C denoting the first three lanthionine rings of nisin; Scheme 1). These truncated nisin molecules can be readily purified with yields in the milligram range, in accordance with protocols reported previously.[26] After purification, they can be modified at the C-terminus by addition of an azide linker. Truncated nisin variants with the azide linker were needed in sufficient quantities for the generation of the semi-synthetic analogues, Therefore, the previously reported peptide coupling procedure was optimized for this study. In order to achieve this optimization, the commonly



Scheme 1. Nisin digestion and semi-synthesis of nisin AB and nisin ABC conjugates. (**a**). Trypsin, Tris buffer, pH 7.0, 30 °C, 48 h; (**b**). Chymotrypsin, Tris buffer, pH 7.5, 30 °C, 48 h; (**c**). Azidopropylamine, PyBOP, DIPEA, DMF, RT, 20 min; (**d**). $CuSO_4$, BTTAA, sodium ascorbate in phosphate buffer, 37 °C, 1 h.

used peptide coupling reagent BOP was used. In a first attempt, azidopropylamine was coupled to nisin AB in the presence of BOP as peptide coupling reagent. However, after performing the reaction under these conditions, substantial amounts of unreacted substrate could be detected by analytical HPLC. Accordingly, the reaction efficiency was a mere 7.4% (Supplementary Figure 1), and prolonging the reaction time to 1 h did not increase the conversion. To optimise the reaction efficiency, PyBOP was used to substitute BOP. After substitution of the coupling reagent, the reaction efficiency increased to 89% (Supplementary Figure 1). For these reactions, PyBOP was shown to be a better coupling reagent than to BOP. Using the optimised conditions from the above experiment, azidopropylamine was coupled to nisin AB and nisin ABC in a reaction containing PyBOP/DIPEA. After 20 minutes, adequate yield was obtained after

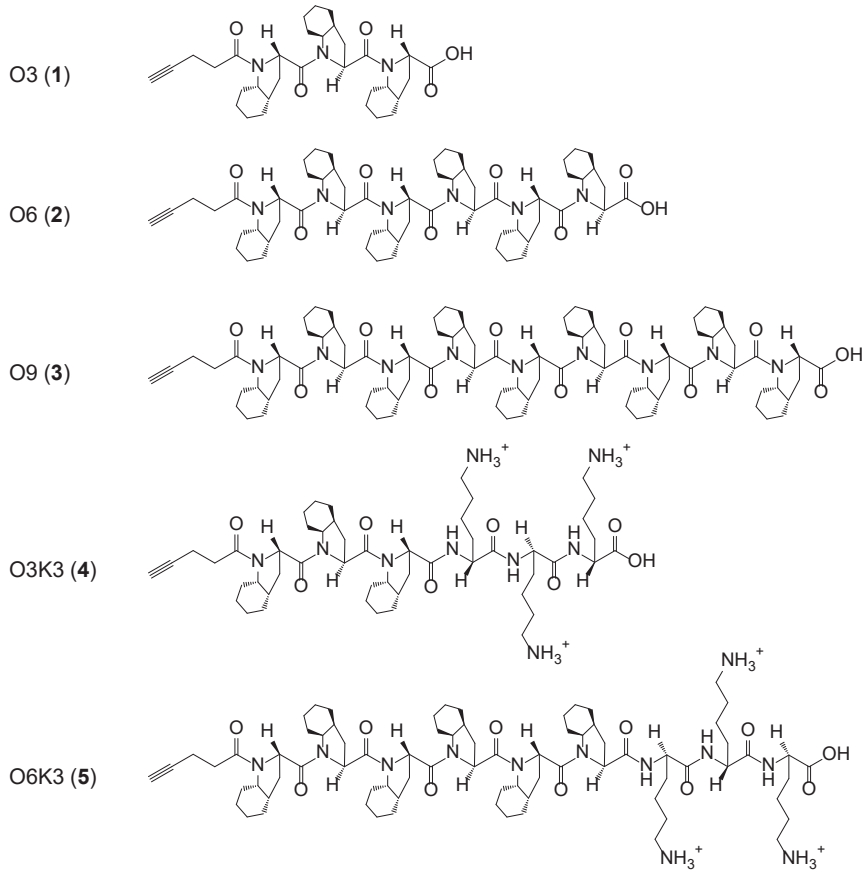


Figure 1. Structure of five hydrophobic pentynoyl peptides O3, O6, O9, O3K3, and O6K3.

which the desired product was purified by HPLC and characterized by MALDI-TOF. The resulting nisin AB-azide and nisin ABC-azide contain convenient handles for ligation to alkynes via CuAAC.

In a first set of experiments, click reactions were performed with nisin AB-azide, nisin ABC-azide and five hydrophobic pentynoyl peptides (**1**–**5**). The first click reaction was attempted with O3 (**1**) and nisin AB-azide in the presence of THPTA as copper (I)-stabilizing ligand. Under this reaction condition, a good amount of product was observed by analytical HPLC. Increasing the reaction temperature to 50 °C and extending the reaction time to 2 h did not increase the conversion, instead it lead to the degradation. Gratifyingly, using BTTAA as substitute of THPTA as copper (I)-stabilizing ligand improved the conversion. Best results were obtained when 9 equiv. O3, 20 equiv. $CuSO_4$, 100 equiv. BTTAA and 1000 equiv. sodium ascorbate were used and reacted at 37 °C for 1 h. Under these optimized reaction conditions, the click reaction

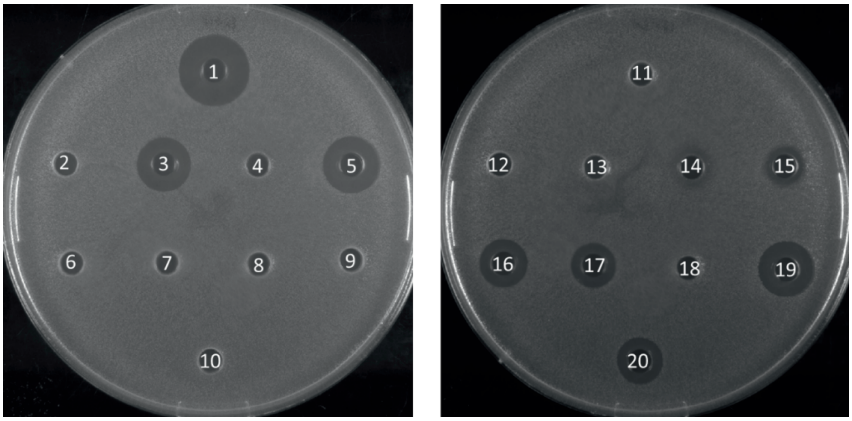


Figure 2. Antimicrobial activity of semi-synthetic nisin analogues against *M. flavus* by agar well diffusion assay. 1: Nisin; 2: Nisin AB; 3: Nisin ABC; 4: Nisin AB-azide; 5: Nisin ABC-azide; 6: O_3; 7: O6; 8: O9; 9: O_3K_3; 10: $O6K_3$; 11: Nisin AB + O_3; 12: Nisin AB + O6; 13: Nisin AB + O9; 14: Nisin AB + O_3K_3; 15: Nisin AB + $O6K_3$; 16: Nisin ABC + O_3; 17: Nisin ABC + O6; 18: Nisin ABC + O9; 19: Nisin ABC + O_3K_3; 20: Nisin ABC + $O6K_3$;

of nisin AB-azide and nisin ABC-azide with the five hydrophobic pentynoyl peptides (**1-5**) were carried out successfully to give semi-synthetic nisin analogues **6-15** in good yields. These semi-synthetic nisin analogues were further characterized by MALDI-TOF.

To investigate the biological activity of nisin analogues, an agar well diffusion assay and a growth inhibition assay were performed. *M. flavus* was used as the indicator strain for the agar well diffusion assay, and 0.15 nmol of each sample was added to each well (Figure 2). The results showed that nisin AB and five hydrophobic pentynoyl peptides (**1-5**) are not active and nisin has the highest activity. Of the nisin AB conjugates, nisin AB + $O6K_3$ is the only active one. Notably, with the exception of nisin ABC + O9, all four nisin ABC conjugates showed activity. Most notably the activity of nisin ABC + O_3K_3 is considerably higher than that of nisin ABC. Antimicrobial activity of all compounds was tested by growth inhibition assays against two clinically relevant Gram-positive pathogens, methicillin resistant *S. aureus* and vancomycin resistant *E. faecium*, as well as *L. monocytogenes*, and *L. lactis*. Their minimal inhibitory concentration (MIC) was determined using an established broth microdilution assay (Table 1), using nisin as a positive control. Nisin AB was devoid of activity at the highest concentration tested except against *E. faecium*. Since of the nisin AB conjugates only nisin AB + $O6K_3$ showed activity, they were only tested against *L. lactis*. In this assay, nisin AB + O9 showed the best activity. Nisin ABC conjugates displayed retained or even increased activity against *E. faecium* and *L. lactis* compared to nisin ABC alone, whereas activity against MW2-MRSA diminished. The antimicrobial activity of nisin ABC + $O6K_3$ against *E. faecium*, *L. monocytogenes* and *L. lactis* decreased only 8-fold,

Table 1. MIC value (μM) of nisin AB and nisin ABC conjugates.

Peptides	MW2-MRSA	*E.faecium*	*L.monocytogenes*	*L.lactis*
Nisin	5.0	0.3	2.5	0.2
O3	>320	>320	>320	>320
O6	>320	>320	>320	>320
O9	>320	>320	>320	>320
O3K3	>160	>160	>160	>160
O6K3	>80	>80	>80	>80
Nisin AB	>320	160	>320	>40
Nisin AB + O3	ND	ND	ND	>40
Nisin AB + O6	ND	ND	ND	20
Nisin AB + O9	ND	ND	ND	5.0
Nisin AB + O3K3	ND	ND	ND	>40
Nisin AB + O6K3	ND	ND	ND	10
Nisin ABC	40	40	40	5.0
Nisin ABC + O3	>80	20	>80	5.0
Nisin ABC + O6	80	5	20	5.0
Nisin ABC + O9	>80	10	40	5.0
Nisin ABC + O3K3	80	40	80	2.5
Nisin ABC + O6K3	80	2.5	10	2.5

Grey: MIC values that are improved in comparison to to one of nisinAB or nisin ABC. ND: not determined.

4-fold, and 12-fold compared to nisin, respectively. Strikingly, its antimicrobial activity against *E. faecium*, *L. monocytogenes* and *L. lactis* increased 16 fold, 4 fold and 2 fold compared to nisin ABC, respectively, and increased all 2 fold compared to nisin ABC + O6, respectively. Compared to nisin ABC, nisin ABC + O6K3 displayed improved activity against *E. faecium*, *L. monocytogenes* and *L. lactis* but decreased activity against MW2-MRSA while nisin ABC + O9 showed enhanced activity against *E. faecium* although activity against other strains was retained or even reduced.

Nisin conjugates

In chapter 4, four methionine analogues were successfully incorporated into different positions of nisin through force feeding (Figure 3A). Methionine analogues like Aha and Hpg (Figure 3B), which contain unique chemical reactive groups (e.g. azide or alkyne), offer the opportunity for post-biosynthetic modifications using biorthogonal synthetic chemistry. In a second set of experiments, click chemistry was performed with six Aha or Hpg containing nisin derivatives (M17I-M21Aha, M17I-M21Hpg, M17I-M21V-M35Aha, M17I-M21V-M35Hpg, M21V-M17Aha, and M21V-M17Hpg), nisin ABC-azide, Cy5-azide and 6-FAM-alkyne according to the optimized protocol described above.

Dimeric nisin constructs and nisin hybrids

The mode of action of nisin involves its binding to lipid II, followed by membrane insertion which leads to pore formation. The pore-complex has a uniform and stable structure, consisting out of eight nisin and four lipid II molecules.[27] Previously, a nisin dimer was prepared by connecting two nisin molecules at the C-terminus through a linker, which led to slightly increased pore-forming activity.[12] As the nisin derivatives prepared for this study contain a clickable group (azide or alkyne) at positions 21, 35, and 17, a setup was devised to investigate how different orientations and multivalency patterns of nisin dimers affect antimicrobial activity.[28] M17I-M21Aha, M17I-M21Hpg, M17I-M21V-M35Aha, M17I-M21V-M35Hpg, M21V-M17Aha, and M21V-M17Hpg were coupled either mutually or with nisin ABC-azide to generate six dimeric nisin constructs and three nisin hybrids which were characterized by MALDI-TOF (Supplementary Figure 2). The antimicrobial activity of these dimers was tested against *M. flavus* by agar diffusion assays. The resulting growth inhibition halos indicated the retainment of at least some degree of activity in all variants. The activity of dimeric nisin constructs M17I-M21Aha + M17I-M21Hpg, M17I-M21V-M35Aha + M17I-M21V-M35Hpg, and M21V-M17Aha + M21V-M17Hpg increased in order as reactions are performed to the hinge region (position 21), the C-terminus (position 35), and ring C (position 17), respectively (Figure 3C and 3D). Coupling M17I-M21Hpg, M17I-M21V-M35Hpg, and M21V-M17Hpg with nisin ABC-azide showed the same antimicrobial activity pattern as the equivalent reactions with M17I-M21Aha, M17I-M21V-M35Aha, and M21V-M17Aha respectively, i.e. activity is altered in ascending order as reactions are performed to the hinge region, the C-terminus, and ring C, respectively (Figure 3D).

Fluorescently labeled nisin variants

Labelling of nisin with fluorescent probes has greatly contributed to understanding its mechanism of action as shown in studies by Scherer et al.[29] and Descobry et al.[30]. The C-terminus of nisin is the common site for labeling. However, introduction of a tag in this position poses a considerable perturbation in the structure and activity of nisin. Here, 6-FAM-alkyne and Cy5-azide (Figure 4A) were successfully coupled at three different positions (positions 21 35, and 17) of nisin which were characterized by MALDI-TOF (Supplementary Figure 3). The antimicrobial activity of six fluorescently labeled nisin variants was retained (Figure 4B), and M21V-M17Aha was found to be the most suitable derivative for labeling with both 6-FAM-alkyne and Cy-5-azide. The localization of six fluorescently labeled nisin variants interacting with *E. faecium* were studied by fluorescence microscopy (Figure 4C). Fluorescence intensity detection indicated that labeled nisin conjugates were all located at the cell membrane. Cy5-azide labeled nisin variants showed lower activity than their 6-FAM-alkyne labeled counterparts, and no aggregation was observed in cell division sites. This may be due to

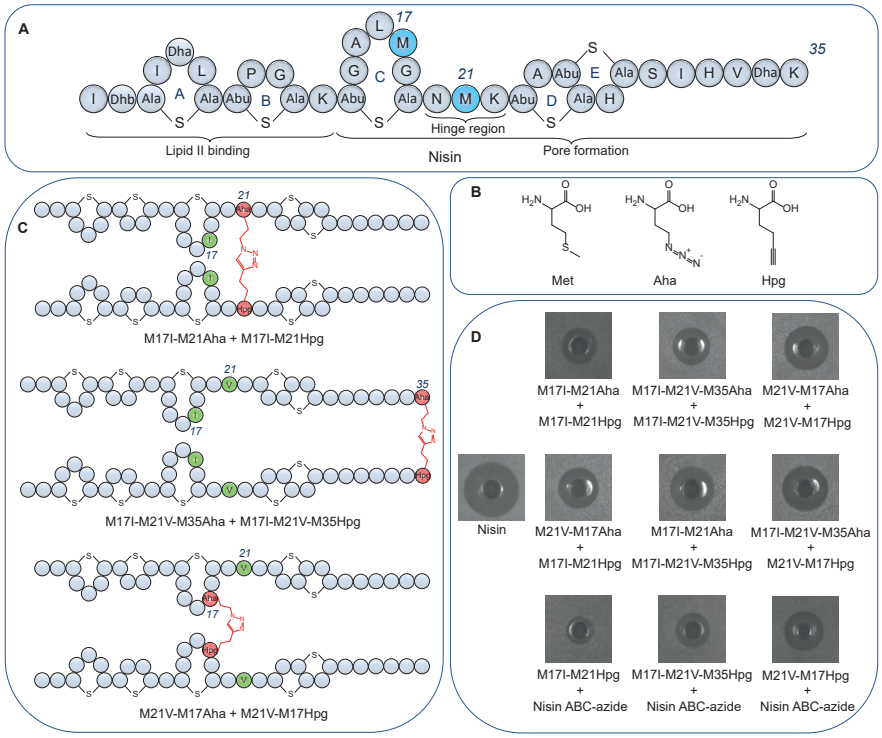


Figure 3. (**A**). Structure of nisin A. Lipid II binding site (rings AB), pore formation domain (rings CDE) and hinge region (NMK) are indicated. Position 17, 21 and 35 which were incorporated methionine analogues of nisin are indicated. Dha: dehydroalanine. Dhb: dehydrobutyrine. Ala-S-Ala: lanthionine. Abu-S-Ala: β-methyllanthionine; (**B**). Structure of methionine (Met) and its analogues (azidohomoalanine (Aha) and homopropargylglycine (Hpg)) used in this study; (**C**). Structure of three representative dimeric nisin constructs with reactions performed at the hinge region (position 21), the C-terminus (position 35), and ring C (position 17). (**D**). Antimicrobial activity of six dimeric nisin and three nisin hybrids at equimolar concentrations against *M. flavus* with nisin as positive control. M17I-M21Aha + M17I-M21Hpg is the least active dimeric nisin construct whereas M21V-M17Aha + M21V-M17Hpg is the most active. Similarly, M17I-M21Hpg + Nisin ABC-azide is the least active nisin hybrid whereas M21V-M17Hpg + Nisin ABC-azide is the most active.

the fact that Cy5-azide would affect the binding of nisin conjugates to lipid II. M21V-M17Aha + 6-FAM-alkyne (Figure 4D) was found to be the most potent fluorescently labeled nisin variant, as it showed similar activity to nisin. It was located at the septum of cell division sites where the membrane-bound cell wall precursor lipid II is maximal. These results are in accordance with previous studies using fluorescently labeled nisin A and nisin Z, which indicated both molecules were accumulating at the cell division sites of *Bacilllus subtilis* and *L.monocytogenes,* respectively.[30,31] M21V-M17Aha + 6-FAM-alkyne showed great potential as a tool to study antibacterial mechanism of action of nisin.

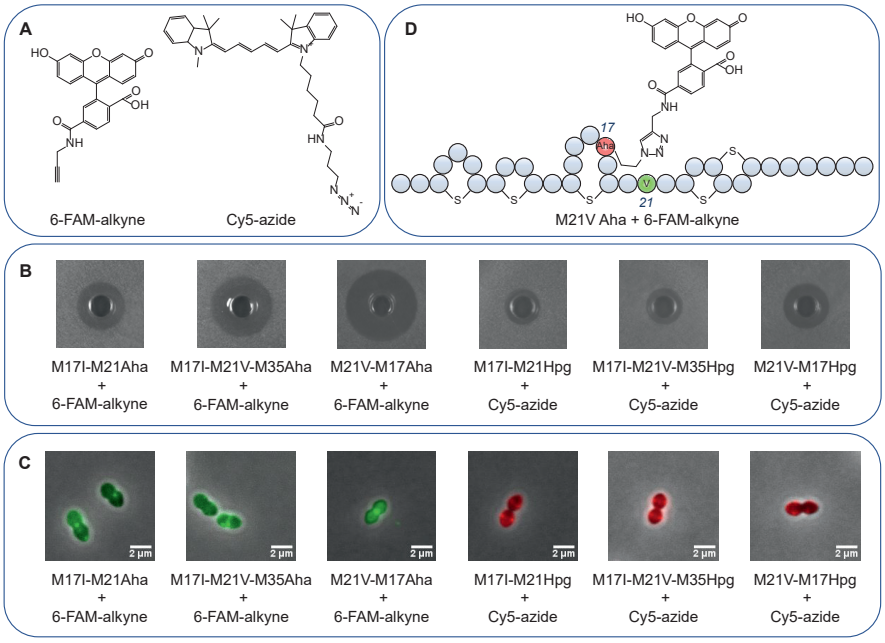


Figure 4. (**A**). Structure of fluorescent dyes 6-FAM-alkyne and Cy5-azide; (**B**). Antimicrobial activity of six fluorescently labeled nisin variants; (**C**). Localization of six fluorescently labeled nisin variants by fluorescence microscopy; (**D**) Structure of the most potent fluorescently labeled nisin variant M21V-M17Aha + 6-FAM-alkyne.

Discussion

In this research, two efficient and direct methods for the preparation of nisin conjugates were developed. For the first approach, nisin AB and nisin ABC was obtained by enzymatic digestion of nisin and these fragments were subsequently C-terminally functionalized with azidopropylamine. Five hydrophobic pentynoyl peptides were synthesized and coupled to nisin AB-azide and nisin ABC-azide by using click chemistry. Ten newly synthesized nisin conjugates were obtained and their antimicrobial activities were tested. The agar diffusion assay showed that the activity of nisin ABC conjugates are much better than nisin AB conjugates, suggesting that ring C is very essential for activity and coupling with nisin ABC is a better choice than nisin AB for modification with these artificial peptides. The growth inhibition experiments showed that the activity of nisin ABC + O6K3 are better than nisin ABC + O6 and nisin ABC + O9, indicating that addition of lysine (positive charge) at the C-terminal region can improve the activity. The antimicrobial activity of nisin ABC + O6K3 against *E. faecium* was 8-fold less active than full-length nisin. However, the activity was 16-fold better than nisinABC, suggesting that modifying nisinABC is a promising strategy to generate semi-synthetic nisin

analogues. In addition, these semi- synthetic nisin analogues showed different inhibition activity in solid media tests compared to the broth MIC test, which can be related to the difference of diffusion ability as the hydrophobicity of five hydrophobic pentynoyl peptides are very different. Importantly, these variants are not prone to degradation at the C-terminus, which has been observed for nisin as it can be degraded by nisinases or other proteolytic enzymes, which could greatly enhance their half-life in the gut.

For the second approach, ncAAs (Aha and Hpg) with reactive groups were incorporated at three different positions of nisin through force feeding. Six dimeric nisin constructs, three nisin hybrids and six fluorescently labeled nisin variants were prepared by using click chemistry and their antimicrobial activity were tested. We found that M17I-M21Aha + M17I-M21Hpg is the least active dimeric nisin construct. Coupling at the hinge region may result in increased steric hindrance and decreased flexibility and therefore hindering its lipid II binding and pore formation features. It again proves that the flexibility of the hinge region is important for the activity which is in accordance with previous studies.[32,33] M17I-M21V-M35Aha + M17I-M21V-M35Hpg showed lower activity. Coupling at C-terminus of nisin gave rise to a dimeric nisin construct contains two lipid II binding sites. However, the pore formation ability may be weakened or abolished as the C-terminus of nisin was involved in the connection. M21V-M17Aha + M21V-M17Hpg is the most active dimeric nisin construct. As we discussed above, ring C is important for the activity. More interestingly, coupling at ring C also give the best activity which may be due to the fact that rings AB are still able to bind lipid II, while the hinge region keeps its flexibility, allowing the C-terminus of nisin to form pores. Coupling nisin ABC-azide, 6-FAM-alkyne, and Cy5-azide at different positions of nisin (21,35, and 17) showed the same activity pattern. The antimicrobial activity of M21V-M17Aha + 6-FAM-alkyne was comparable to nisin and it can be used as a model to investigate the mechanism of action and for understanding the mechanism of synergistic of nisin with other molecules on Gram-negative strain.

Two methods employed in this study for coupling moieties with lantibiotic via click chemistry are in many ways complementary to one another. For lantibiotics that are available commercially, enzymatic digestion followed by attaching a functional group is a good option to modify and it is easy to perform and highly efficient. However, it is only feasible when the fragment contains a single carboxylate such as nisin. Most lantibiotics contain multiple carboxylates which make it more difficult to label. Incorporation ncAAs (e.g. Aha and Hpg) with reactive groups into lantibiotics provides a means to modify such peptides. It has the advantage of freedom of design as the ncAAs can be incorporated at any position of lantibiotics and the lantibiotics can be therefore coupled to desired moeities at any position. Overall, this study highlights how lantibiotics can be used as lead structures to create novel variants with altered properties (e.g. stability, activity, and specificity) via chemical coupling.

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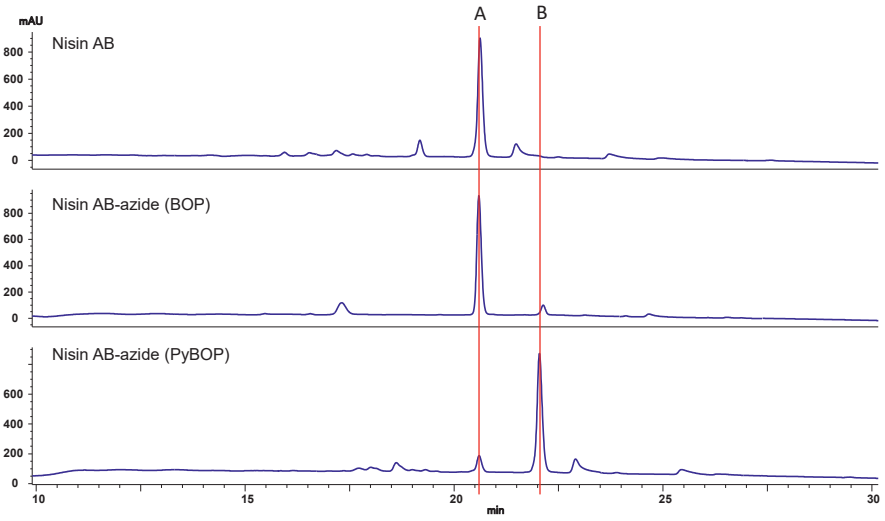
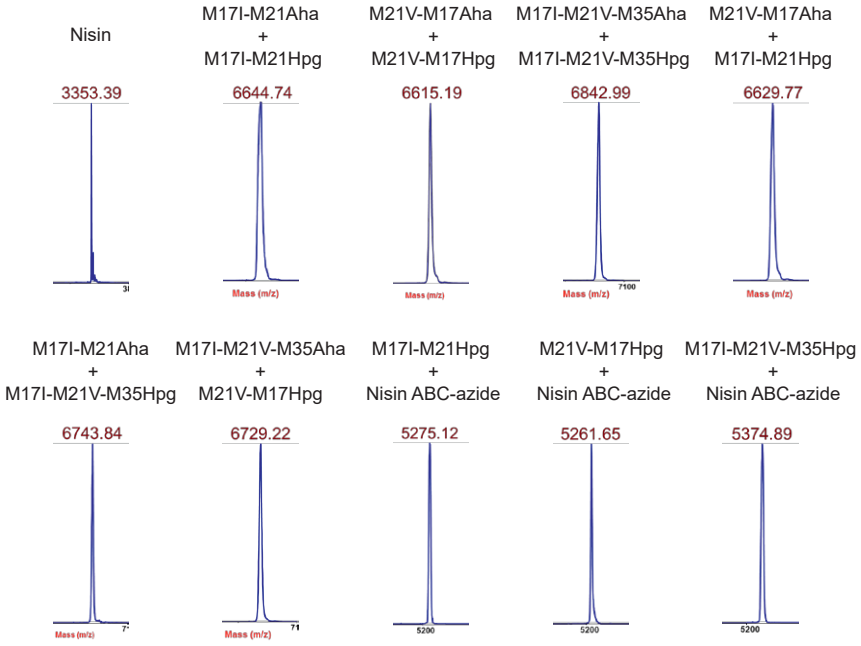
Suplementary Figures



Supplementary Figure 1: HPLC analysis of reaction substrate nisin AB and the reaction mixtures using BOP and PyBOP. A: peak of substrate; B: peak of product.



Supplementary Figure 2. MALDI-TOF analysis of nisin, demeric nisin cosntructs and nisin hybrids.

Supplementary Figure 3. MALDI-TOF analysis of fluorescently labeled nisin variants.

CHAPTER 6

Summary and general discussion

Antimicrobial resistance is one of the greatest threats to global health nowadays and it has a significant impact on global health and economy throughout the world.[1] According to a new and groundbreaking report released by UN Ad hoc Interagency Coordinating Group on Antimicrobial Resistance in 2019, if no action is taken, antimicrobial resistance could cause 10 million deaths each year by 2050 and force up to 24 million people into extreme poverty by 2030.[2] Research and development for new technologies to combat antimicrobial resistance are urgently needed. Peptide-based therapeutics have gained greatly increased interest during recent years due to its high selectivity, efficacy, tolerability and excellent safety.[3] Currently, more than 400 peptide drugs are under global clinical developments and over 60 peptide drugs have been approved for clinical use.[4] Ribosomally synthesized and post-translationally modified peptides (RiPPs) represent an important class of gene-coded peptides with extensive post-translational modifications.[5] Among RiPPs, the class of lanthipeptides represents a rich source for promising leads against Gram-positive bacteria.[5] Lanthipeptides possessing antimicrobial activity are called lantibiotics which contains unusual post-translationally modified amino acid residues such as dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionines (Lans) and methyllanthionines (MeLans), that are introduced by a promiscuous post-translational modification (PTM) machinery.[6] The unique biosynthetic pathways and relatively low genetic complexity of biosynthesis make lantibiotics good candidates for synthetic biology and bioengineering to expand the antimicrobial arsenal. Several lantibiotics have been considered as lead structures for therapeutic use[7-9] and a number of lantibiotics (e.g. NAI-107, mutacin 1140, duramycin, and NVB302) have entered preclinical development and clinical trials.[10-12] In this thesis, various strategies for lanthipeptides engineering were employed to produce novel antimicrobials (**Chapter 3, Chapter 4, and Chapter 5**).

Large-scale engineering of lanthipeptides could be a promising strategy to obtain novel bioactive variants, but a general challenge is a lack of efficient methods to explore the antimicrobial activity of large number of variants. **Chapter 3** presents a powerful approach to generate topologically novel antimicrobial lanthipeptides by modular bioengineering of lanthipeptide modules. By combinatorial shuffling of 33 lanthipeptide modules with natural or synthetic background, a library of 6,000 putatively active structures was obtained. The nanoFleming platform, a miniaturized and parallelized high-throughput inhibition assay, was developed by ETH collaborators to enable rapid bioactivity assessment of the library peptides by evaluating the result of co-culturing lanthipeptides producers and a sensor strain at nanoliter scale in nanoliter reactors at high-throughput. Based on a hit-set of over 100 molecules, lanthipeptide variants with improved activity against pathogenic bacteria and altered activity spectrum were identified. Nonetheless, this nanoFleming platform has some drawbacks that can be improved in the future. To some extent, the substrate specificity of the NisBTC limited

the diversity of peptides that can be produced. Future approaches might include various different PTM enzymes co-expressed in the production host or even include direct evolution on such enzymes to broaden their substrate specificity and therefore broaden the diversity of peptides. In the future, this platform might not only be used for the discovery of molecules with improved or altered activity spectrum but also for the generation of sufficient diversity required at other steps in drug development of peptides (e.g. to test candidate peptides for plasma stability or activity *in vivo*).

Another efficient method to broaden the structure diversity and functionalities of lantibiotics is incorporation of noncanonical amino acids (ncAAs).[13-16] ncAAs represent a highly diverse pool of building blocks which can offer unique physicochemical properties and chemical handles.[17] Residue and site-specific methods for incorporation ncAAs in several production hosts hold a great potential for the generation of novel antimicrobials with desirable properties.[18,19] In **Chapter 4**, we demonstrated for the first time the incorporation of methionine analogues into RiPPs in *Lactococcus lactis*. The class I model lantibiotic nisin was chosen for this study. Four methionine analogues with unsaturated and varying side chain length were successfully installed into four distinct positions of nisin. The amino acid replacement and incorporation efficiency of ncAAs into nisin derivatives was verified and the results showed that azidohomoalanine (Aha) and homopropargylglycine (Hpg) are excellent methionine surrogates. The growth inhibition experiments revealed that replacement of Met with Met analogues with different properties can alter the antimicrobial activity spectrum. For example, M17Aha-M21Aha showed strongly reduced activities against several strains, but activity against *L. monocytogenes* was improved. Our experiments further exemplify one of the most important applications of ncAA incorporation, that is, the structural and chemical diversification of RiPPs. As *L. lactis* is also autotrophic for leucine, isoleucine, valine and histidine, more ncAAs can be incorporated through this system in the futrue. Future studies might also include introducing other PTM enzymes co-expressed in this system to further broaden the diversity of lantibiotics or constructing a new expression system with class II PTM to facilitate the incorporation of ncAAs into other type of lantibiotics. In addition, methionine analogues that possess chemical handles (e.g. Aha and Hpg) provides the opportunity of chemical coupling using a variety of ligands such as fluorophores and peptide moieties via copper (I)-catalyzed click chemistry.

Subsequently in **Chapter 5**, nisin derivatives possessing Aha and Hpg at position 17, 21 or 35 generated in work described in **Chapter 4** were coupled either mutually or with nisin ABC-azide , Cy5-azide and 6-FAM-alkyne to obtain six dimeric nisin constructs, three nisin hybrids and six fluorescently labeled nisin variants via click chemistry. We found that the activity of dimeric nisin constructs increased in order as reactions are performed to the hinge region (position 21; Figure 1B), the C-terminus

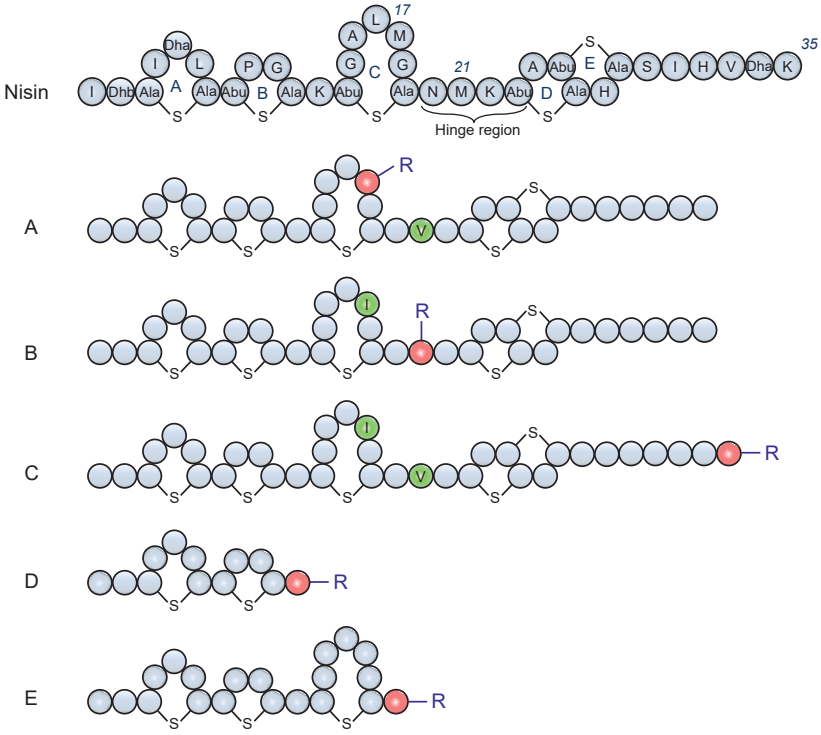


Figure 1. Structure of nisin and design of coupling nisin derivatives with moieties. R, the coupling moieties; In Red, position for coupling; In green, Met residues replaced by Ile or Val.

(position 35; Figure 1C), and ring C (position 17; Figure 1A). The same activity pattern was observed when nisin derivatives were coupled with nisinABC-azide, Cy5-azide, or 6-FAM-alkyne. It again revealed that the flexibility of the hinge region is important for the activity which is in accordance with previous studies.[20,21] Coupling at the C-terminus may lead to the abolishment or weakening of pore formation ability, which results in lower activity. Interestingly, coupling at ring C give the best activity which may be due to the fact that rings AB are still able to bind lipid II, while the hinge region keeps its flexibility, allowing the C-terminus of nisin to form pores. The C-terminus of nisin is the common site for labelling with fluorescent probes.[22] However, introduction of a tag in this position poses a considerable perturbation in the structure and activity of nisin. In this chapter, the fluorescent dyes 6-FAM-alkyne and Cy5-azide were coupled at position 17, 21 and 35, respectively. M21V-M17Aha + 6-FAM-alkyne was found to be the most potent fluorescently labeled nisin variant as it showed comparable activity to nisin and the fluorescence intensity detection indicated that it was located at the septum of cell division sites which is in accordance with previous studies.[22,23] Therefore, M21V-M17Aha + 6-FAM-alkyne is the most suitable fluorescently labeled

nisin variant for studying the mechanism of action and it can also be used to investigate the mechanism of synergistic action of nisin with other antibiotics on Gram-negative strains. This strategy can be extended to modify other RiPPs. With numerous novel RiPPs reported, little is known about the mechanism of action of these peptides. It would be highly appropriate to use this method to modify such RiPPs with biomarkers or fluorescence probes to investigate their mechanism of action. Overall, this study suggests that the bioorthogonal reactive groups of ncAAs can serve as a platform for post-biosynthetic modifications, such as conjugating with peptides, or functional labels (e.g. fluorescence). The insertion of ncAAs during translation along with the possibility for their subsequent modification (post-synthetic conjugation) offers additional chemical and structural diversity of the generation of novel RiPPs. With a general increase in chemical diversity, we expect to provide peptide structural scaffolds with, for example, enhanced resistance to degradation, or increased bioavailability and eventually be able to overcome the disadvantages that are usually associated with peptides as drug candidates.

Moreover, **Chapter 5** describes another method for the preparation of nisin conjugates via click chemistry. Nisin AB and nisin ABC (A, B and C denoting the first three lanthionine rings of nisin; Figure 1D and E), obtained from enzymatic digestion of nisin, were C-terminally functionalized with azidopropylamine to generate nisin AB-azide and nisin ABC-azide which were subsequently coupled with five hydrophobic pentynoyl peptides obtained from Dr. Kubyshkin by using click chemistry. Ten newly synthesized nisin conjugates were obtained and their antimicrobial activities were tested. The agar diffusion assay showed that the activity of nisin ABC conjugates are much better than nisin AB conjugates, suggesting that ring C is quite essential for activity and nisin ABC is a better candidate than nisin AB for modification with these artificial peptides. The activity of nisin ABC + O6K3 against against *E. faecium* decreased only 8- fold compared to nisin. Strikingly, its antimicrobial activity against *E. faecium* was 16- fold better than nisin ABC, suggesting that modifying nisinABC is a promising strategy to generate semi-synthetic nisin analogues. Importantly, these variants are not prone to degradation at the C-terminus, which has been observed for nisin as it can be degraded by nisinases or other proteolytic enzymes, which could greatly enhance their half-life in the gut. In this chapter, nisin AB-azide and nisin ABC-azide can be readily generated with yields in the milligram range according to our optimized protocol. Future studies may focus on coupling peptides, especially anti-Gram-negative peptides, with nisin ABC-azide.

The two methods employed in **Chapter 5** for coupling moieties to a lantibiotic via click chemistry are in many ways complementary to one another. Enzymatic digestion of lantibiotics followed by attaching a functional group at the C-terminus is only feasible when the fragment contains a single carboxylate. This method is easy to

perform and highly efficient and the peptides might be generated in high yield with the optimized protocol. However, several lantibiotics possess more than one carboxylate which make it more difficult to modify. Incorporation ncAAs with reactive groups (e.g. alkyne and azide) into lantibiotics provides a means to modify such peptides. Since ncAAs can be incorporated at any position of a lantibiotic, the lantibiotics can also be coupled at desired positions. On the other hand, genetic manipulation of the target sequence might be required and the production yield of desired peptides might be highly different. Overall, this chapter highlights how lantibiotics can be used as lead structures to create novel variants with altered properties (e.g. stability, activity, and specificity) via chemical coupling.

Chapter 2 investigates the specificity and application of the lantibiotic protease NisP. Two sets of nisin variants were constructed to test the ability of NisP to cleave leaders from various substrates. The first set was designed to study the influence of variations in the leader peptide or variations around the cleavage site. The second set was designed to investigate the influence of the lanthionine ring topology. The results suggest that NisP is the most suitable and inexpensive protease for the activation of diverse lantibiotics or thioether-stabilized peptides, produced with the nisin leader peptide and the modification machinery of nisin among all the proteases tested. The presence of lanthionine rings is not mandatory for the cleavage. These insights should help to expand the biotechnological potential of NisP as a general tool for the cleavage of proteins with and without lanthionine residues. In addition, NisP generated in work described in **Chapter 2** was used to activate diverse lanthipeptides in **Chapter 3**, **Chapter 4**, **and Chapter 5**.

In summary, three different strategies: i) large-scale modular engineering aided by nanoFlaming screening, ii) incorporation of ncAAs, and iii) chemical coupling were employed to develop novel antimicrobials. These approaches are able to change the structure and chemical diversity of lanthipeptides and expanded our understanding of structure-activity relationship, and have also led to the development of lantibiotic derivatives with enhanced functionality in terms of activity spectrum, stability and specific activity against clinical relevant antibiotic-resistant pathogens. It is thus likely that lantibiotics one day can be used as part of the antimicrobial arsenal to combat antimicrobial resistance.

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Nederlandse samenvatting

Antimicrobiële resistentie is tegenwoordig één van de grootste bedreigingen voor de wereldgezondheid en heeft een aanzienlijke invloed op de gezondheid en economie overal ter wereld. Onderzoek en ontwikkeling van nieuwe technologieën om antimicrobiële resistentie te bestrijden zijn dringend nodig. De interesse in therapieën gebaseerd op peptiden is de laatste jaren sterk toegenomen vanwege de hoge selectiviteit, werkzaamheid, verdraagbaarheid en uitstekende veiligheid. Ribosomaal gesynthetiseerde en posttranslationele gemodificeerde peptidens (RiPPs) vertegenwoordigen een belangrijke klasse van gen-gecodeerde peptiden met uitgebreide posttranslationele modificaties. Onder RiPPs vertegenwoordigt de klasse van lanthipeptiden een rijke bron voor veelbelovende antimicrobiële peptiden tegen grampositieve bacteriën. De unieke biosynthetische routes en de relatief lage genetische complexiteit van biosynthese maken lantibiotica goede kandidaten voor synthetische biologie en bio-engineering om het antimicrobiële arsenaal uit te breiden. In dit proefschrift werden verschillende strategieën voor lanthipeptide-engineering gebruikt om nieuwe antimicrobiële stoffen te produceren (**hoofdstuk 3, hoofdstuk 4** en **hoofdstuk 5**).

Hoofdstuk 3 beschrijft de modulaire bio-engineering van antimicrobiële lanthipeptiden met behulp van nanoFleming-screening, een geminiaturiseerde en parallelle high-throughput inhibitietest ontwikkeld door ETH-medewerkers. Door combinatoriele herschikking van 33 lantibiotica modules afkomstig van 12 antimicrobiële lanthipeptiden en 4 synthetische peptiden, werd een bibliotheek van 6000 vermoedelijk actieve structuren gegenereerd. Screening van de bibliotheek met het nanoFleming-platform gevolgd door karakterisering resulteerde in 11 antimicrobiële lanthipeptiden die verbeterde antimicrobiële activiteit vertoonden in vergelijking met de wildtype peptiden of die in staat waren om resistentiemechanismen te omzeilen.

In **hoofdstuk 4** hebben we voor het eerst de opname van methionine-analogen in RiPP's in *Lactococcus lactis* aangetoond. Voor dit onderzoek werd nisine gekozen wat een model peptide is van klasse I lantibiotica. Vier methionine analogen met onverzadigde en variërende zijketenlengte werden met succes geïntroduceerd in vier verschillende posities van nisine. De aminozuurvervanging en opname efficiëntie van non-canonical amino acids (ncAAs) in nisine derivaten werd geverifieerd en de resultaten toonden aan dat azidohomoalanine (Aha) en homopropargylglycine (Hpg) uitstekende methionine surrogaten zijn. De inhibitietesten onthulden dat vervanging van methionine (Met) door Met-analogen met verschillende eigenschappen het antimicrobiële activiteitenspectrum kan veranderen.

Vervolgens werden in **hoofdstuk 5** twee efficiënte en directe methoden ontwikkeld voor de bereiding van nisine-conjugaten via zogenaamde klikchemie. In de eerste methode werden afgeleiden van nisine gebruikt, die reactieve groepen bevatten (d.w.z.

alkyn of azide) en voortgekomen zijn uit het werk van hoofdstuk 4,om vervolgens nisine te verbinden met peptidegroepen en fluorescerende probes. Zes dimere nisine constructen, drie nisine hybriden en zes fluorescerende nisine varianten werden ontwikkeld en hun antimicrobiële activiteit bleef behouden, wat de kracht van deze benadering laat zien als hulpmiddel voor het bestuderen van de lokalisatie en het werkingsmechanisme van nisine. In de tweede benadering werden C-terminaal gefunctionaliseerde nisine AB en nisine ABC geconjugeerd met vijf hydrofobe pentynoylpeptiden van Dr. Kubyshkin. De resulterende semi-synthetische nisine analogen vertoonden krachtige remming van bacteriegroei.

In hoofdstuk 2 werd de specificiteit en toepassing van de lantibiotica protease NisP bestudeerd. Twee groepen nisine varianten werden gemaakt om het vermogen van NisP te testen om de leider sequentie van verschillende substraten te splitsen. De eerste groep werd ontworpen om de invloed van variaties in de leider peptide of variaties rond de splitsingsplaats te bestuderen. De tweede groep werd ontworpen om de invloed van de lanthionine-ringtopologie te onderzoeken. Deze studie suggereert dat NisP een grotere substraattolerantie heeft dan eerder werd gedacht en dat NisP is een geschikte en goedkope protease voor de activering van diverse lantibiotica die heteroloog tot expressie zijn gebracht.

Samenvattend, drie verschillende strategieën: i) grootschalige modulaire engineering ondersteunt door nanoFlaming screening, ii) incorporatie van ncAAs, en iii) chemische koppeling werden gebruikt om nieuwe antimicrobiële stoffen te ontwikkelen. Deze benaderingen zijn in staat om de structuur en chemische diversiteit van lanthipeptiden te veranderen en ons begrip van structuur-activiteit relaties te vergroten. Ook hebben de strategieën geleid tot de ontwikkeling van lantibiotica derivaten met verbeterde functionaliteit in termen van activiteitsspectrum, stabiliteit en specifieke activiteit tegen klinisch relevante antibioticaresistente pathogenen. Het is dus waarschijnlijk dat lantibiotica op een dag gebruikt kunnen worden als onderdeel van het antimicrobiële arsenaal om antimicrobiële resistentie te bestrijden.

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邓晶晶

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List of Publications

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3. Montalban-Lopez, M.* **Deng, J.***, van Heel, A. & Kuipers, O. P. (**2018**). Specificity and application of the lantibiotic protease NisP. *Frontiers in microbiology* 9:160, doi: 10.3389/fm icb.2018.00160.
4. **Deng, J.** & Lu, C. (**2015**). Two new isoflavone 7-O-α-4″-anhydro-4″,5″-didehydro-glucuronides from *Streptomyces* sp. LZ35 Δ*gdmAI*. *Natural product research* 30 (2), 1–5, doi: 10.1080/14786419.2015.1046869.
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